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3′-end labeling of RNA with recombinant yeast poly(A) polymerase

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ABSTRACT

Two commonly used methods to end-label RNA-molecules are 5′-end labeling by polynucleotide kinase and 3′-end labeling with pCp and T4 RNA ligase. We show here that RNA 3′-ends can also be labeled with the chain-terminating analogue cordycepin 5′-triphosphate (3′-deoxy-ATP) which is added by poly(A) polymerase. For a synthetic RNA it is shown that 40% of cordycepin becomes incorporated when the nucleotide is used at limiting concentrations and that with an excess of cordycepin 5′-triphosphate essentially all the RNA becomes modified at its 3′-end. The reaction is complete within minutes and the RNA product is uniform and suitable for sequence analysis. The efficiency of labeling varies with different RNA-molecules and is different from RNA ligase. Poly(A) polymerase preferentially labels longer RNA-molecules whereas short RNA-molecules are labeled more efficiently by T4 RNA ligase.

INTRODUCTION

Poly(A) polymerases catalyze the addition of adenosine 5′-monophosphate residues from ATP to the 3′ terminal hydroxyl group of any oligo- or polyribonucleotide. Poly(A) polymerase from *Escherichia coli* has been used in the past for the end-labeling of tRNAs (1,2). However, this enzyme is not available in pure form and is difficult to prepare free of contaminating nucleases. In contrast, highly purified yeast poly(A) polymerase is easily obtained in large amounts from recombinant *E.coli* (3) and can be used for the modification of any RNA at its 3′-end *in vitro*. Several hundred adenosine residues can be added to RNA 3′-ends. Because the reaction occurs in a distributive mode (4), a population of adenylated RNA molecules is generated that are similar in length. If instead of adenosine 5′-triphosphate the chain-terminating analogue cordycepin 5′-triphosphate (3′-deoxyadenosine 5′-triphosphate) is used in the reaction, only a single cordycepin molecule is added to the RNA and the 3′-end is blocked for further extension because of the lack of a 3′ OH. This reaction can be written as follows:

$$RNA\text{-}_{3'OH} + \text{cordycepin 5′-triphosphate} \rightarrow RNA\text{-}pA\text{-}_{3'H} + PP_i$$

When the α-phosphate of cordycepin 5′-triphosphate is labeled with ^{32}P, a product RNA is generated that is extended by one nucleotide and radioactively labeled at its 3′-end. As shown below, the reaction is very fast and efficient even at low RNA concentrations. The 3′-end-labeled RNA is homogeneous in length and is therefore suited for chemical or enzymatic sequence analysis. This method of labeling RNA 3′-ends complements the use of polynucleotide kinase to label RNA 5′-ends and of RNA ligase to label 3′-ends with pCp (5,6).

MATERIALS AND METHODS

Poly(A) polymerase

The T7 overexpression system of Studier (7) was used for expression of yeast poly(A) polymerase in *E.coli*. Enzyme activity was assayed as described (4). Plasmid pJPAP1 (3) was used to transform BL21(DE3) pLysS. This strain contains the T7 RNA polymerase gene under the control of the *lac* promotor on the chromosome and carries the T7 lysozyme gene on pLysS. Cells were grown at 37°C to an OD_{600} of 0.6 in 2 l of Luria broth supplemented with 30 μg/ml chloramphenicol and 100 μg/ml ampicillin. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to 1 mM and cells were grown overnight at 18°C on a shaker. Cells were harvested by centrifugation at 4°C at 4000×g for 15 min. All subsequent steps were carried out at 0°C–4°C. The pellet was resuspended in 20 ml 222 mM KCl, 55.5 mM Tris–HCl, pH 8.0, 1.1 mM DTT, 2.2 mM EDTA and frozen in liquid nitrogen. Upon thawing, the cells lysed completely due to the presence of T7 lysozyme. Glycerol was added to 10% and the lysate was sonicated six times with ten second pulses and cooling in between on ice. The lysate was centrifuged at 15,000×g for 30 min and again at 300,000×g for 2 h. The supernatant was diluted to final concentrations of 100 mM KCl, 50 mM Tris–HCl pH 8.0, 0.5 mM DTT, 1 mM EDTA, 10% glycerol and loaded onto a 100 ml DEAE-Sepharose (Pharmacia) column equilibrated in the same buffer. The flow-through, which contained all of the poly(A) polymerase activity, was collected, $CaCl_2$ was added to 1 mM, and the pool was loaded onto a 20 ml hydroxyapatite (IBF Biotechnics, Villeneuve-la-Garenne, France) column in 20 mM Tris–HCl, pH 8.0, 50 mM KCl, 10%

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glycerol and 0.5 mM DTT. The column was washed with one column volume of loading buffer, one column volume of loading buffer containing 2 M NaCl and again with one column volume of loading buffer and eluted with a 200 ml gradient from 20 mM Tris−HCl, pH 8.0 to 500 mM potassium phosphate, pH 7.9 in 50 mM KCl, 10% glycerol and 0.5 mM DTT. Poly(A) polymerase, desorbed at 130 mM potassium phosphate, was dialyzed overnight against 50 mM KCl, 20 mM HEPES-KOH pH 7.0, 0.2 mM EDTA, 0.5 mM DTT, 10% glycerol and loaded onto a 1 ml Mono S-FPLC column (Pharmacia) equilibrated in the same buffer. The column was eluted with a 40 ml gradient from 50 to 500 mM KCl in the same buffer at 0.5 ml/min. Poly(A) polymerase eluted at 250 mM KCl. About 3 mg of pure poly(A) polymerase with a specific activity of 1.3×10^6 units/mg was obtained. The recovery was 35%. One unit of enzyme corresponds to 1 pmol AMP added to poly(A) per minute (4).

No loss of activity was observed when poly(A) polymerase was stored for six months at −20°C in 20 mM Tris−HCl, pH 8.0, 60 mM KCl, 0.2 mM EDTA, 1 mM DTT, 0.02% NP40, 50% glycerol (P. Preker, personal communication).

The preparation of poly(A) polymerase was nuclease free. A 20 min incubation with poly(A) polymerase under reaction conditions did not result in any degradation of *L3pre* RNA (3′-end labeled with cordycepin) as determined by gel electrophoresis (data not shown).

The expression vector pJPAP1 is available from the authors by request. Homogeneous recombinant yeast poly(A) polymerase can be purchased from United States Biochemical Corp. (Cleveland, Ohio).

RNA

L3pre RNA was synthesized *in vitro* with plasmid BST7 L3 as template (8). Plasmid DNA was cleaved with *RsaI* and transcribed with T7 RNA polymerase (Boehringer) as described (9). *L3pre* RNA has the sequence 5′-GGGCGAAUUGGGGUCACUAGG-AGACACUUUCAAUAAAGGCAAAUGUUUUU-AUUUGU-3′.

HeLa cell nuclear extract and Mono Q-FPLC fractions enriched for U1 snRNPs and U2 snRNPs respectively (10), were obtained from A.Krämer. RNA was extracted from these fractions as described (10) and kept under ethanol.

Labeling and RNA sequencing

RNA was 3′-end labeled with cordycepin 5′-triphosphate by poly(A) polymerase at 30°C in 10−50 μl containing 25% 4×buffer (= 80 mM Tris−HCl, pH 7.0, 40% glycerol, 200 mM KCl, 2.8 mM $MnCl_2$, 0.8 mM EDTA), 0.1 mg/ml acetylated BSA, 0.2 μM RNA (variable), 0.04 μM cordycepin 5′-triphosphate (α-^{32}P labeled, 10 mCi/ml, 5000 Ci/mmol, NEN), 20 μM cordycepin 5′-triphosphate diluted from a 25 mM stock solution in 0.1 M Hepes-KOH, pH 7.9 (Boehringer Mannheim; only for complete 3′-end blocking), 50000 units/ml poly(A) polymerase.

pCp-labeling of RNA (5) was carried out overnight at 4°C in 20 μl containg 50 mM Hepes-KOH, pH 7.5, 3.3 mM DTT, 20 mM $MgCl_2$, 5 μM ATP, 10 μg/ml acetylated BSA, 10% DMSO, 1 μl cytidine 3′, 5′- [5′-^{32}P] biphosphate (10 mCi/ml, 3000 Ci/mmol, Amersham), 4 units T4 RNA ligase (New England Biolabs), and 15 units RNA guard (Pharmacia). Labeled RNA was purified by extraction with phenol and chloroform and precipitated with ethanol.

Sequencing of 3′-end labeled RNA was carried out chemically (11) exactly as described (12).

RESULTS

Labeling of a synthetic RNA with cordycepin triphosphate

The parameters which influence the activity of poly(A) polymerase *in vitro* have been described earlier (2). Poly(A) polymerase is specific for the extension of RNA molecules as opposed to DNA molecules and for the incorporation of adenosine nucleotides. When ATP was replaced by [α-^{32}P] cordycepin 5′-triphosphate, denaturing polyacrylamide gel electrophoresis revealed labeling of the RNA substrate, i. e. cordycepin incorporation. In order to optimize 3′-end labeling of an individual RNA (*L3pre*), time-course experiments were carried out under two different conditions. To label the RNA to high specific activity, no unlabeled cordycepin 5′-triphosphate was included in the reaction (Figure 1A), whereas for the complete

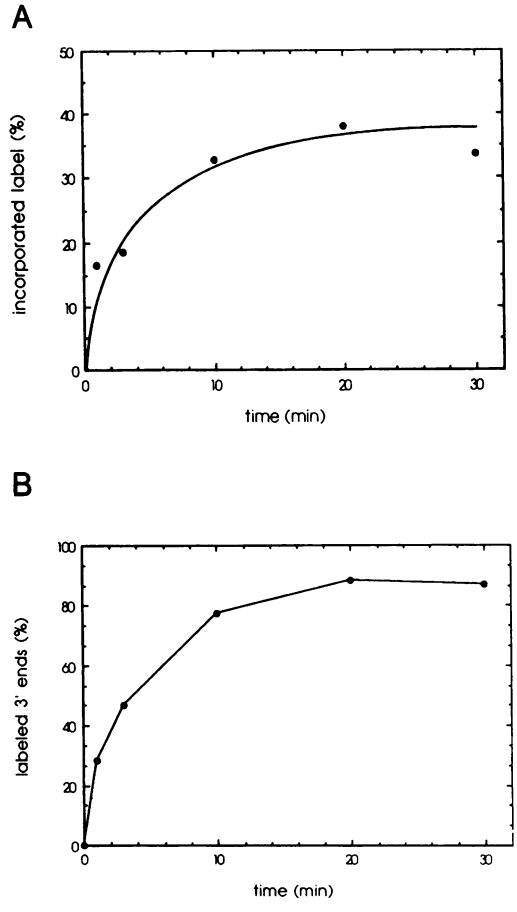

Figure 1. Kinetics of 3′-end labeling of *L3pre* RNA. *L3pre* RNA (0.2 μM) was synthesized as described in Materials and Methods and labeled at the 3′-end with cordycepin 5′-triphosphate. Aliquots were removed from the reaction after different time points and loaded directly on a 6% polyacrylamide gel after addition of formamide loading buffer. The efficiency of labeling (ordinate) was quantified by counting gel slices containing the labeled RNA in a scintillation counter. *L3pre* RNA was quantified by measuring the OD at 260 nm. **(A)** Labeling under conditions that allows high efficient 3′-end labeling i. e. with limiting amounts (0.04 μM) of cordycepin 5′-triphosphate. **(B)** Labeling under conditions that allow highly efficient 3′-end blocking i.e. with an excess (20 μM) of cordycepin 5′-triphosphate.

blocking of the 3′-end of the RNA, an excess of unlabeled cordycepin 5′-triphosphate was included (Figure 1B). Incorporated label was quantified at different time points as indicated in the Figure legend. In the reaction with limiting amounts of cordycepin 5′-triphosphate maximum incorporation (30−40%) was reached after 10−20 min. In the reaction with an excess of cordycepin 5′-triphosphate i.e. under conditions that ensure complete 3′-end blockage, 78% of the 3′-ends were modified after 10 minutes and essentially all of the RNA was blocked after 20 minutes by the addition of a cordycepin 5′-monophosphate.

The 3′-end of labeled RNA is homogeneous

As a test of the homogeneity of the labeled 3′-end, the *L3pre* RNA was subjected to chemical sequence analysis (Figure 2). After a limited modification with diethylpyrocarbonate (A+G modification) or hydrazine (C+U modification), respectively, the RNA was cleaved with aniline and the reaction products were separated on a 12% polyacrylamide gel. The analysis of the sequencing ladder revealed that the labeled RNA was uniform and of high quality since no degradation and sequence contamination was detectable.

The efficiency of labeling is influenced by RNA sequence and length

For a qualitative comparison of 3′-end labeling with poly(A) polymerase and RNA ligase, respectively, RNA was isolated from HeLa cell nuclear extract and from chromatographic fractions enriched for U1 and U2 snRNA (10). Isolated RNA was separated on a denaturing 10% polyacrylamide gel and visualized by staining with ethidium bromide (Figure 3A). The same RNA fractions were also labeled at the 3′-end, either with cordycepin 5′-triphosphate by poly(A) polymerase or with pCp by RNA ligase and separated on a 10% polyacylamide gel (Figure 3B). This analysis reveals that poly(A) polymerase and RNA ligase have different substrate preferences. A comparison with the ethidium bromide stained gel shows further that neither of the two labeling methods accurately reflects the amount of individual RNA molecules present in the RNA preparations although one has to consider that ethidium bromide stains RNA not only in proportion to molar amount and length, but is influenced by secondary structures as well. However, as can be seen with RNA isolated from HeLa cell nuclear extract (Figure 3B, lanes 1 and 2) poly(A) polymerase labels long RNA molecules very efficiently whereas RNA ligase has a pronounced preference for short RNA molecules. In addition to the length of the RNA substrate, the sequence and structure seem to play some role as well. U2 snRNA is a very good substrate for poly(A) polymerase whereas U1 snRNA is labeled much less efficiently. RNA ligase, in contrast, has a less pronounced preference for

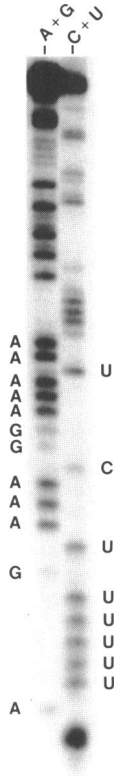

Figure 2. Chemical sequencing of *L3pre* RNA that was labeled with cordycepin at the 3′-end. RNA was sequenced as described (12). Aliquots were separated on a 12% sequencing gel. Left lane: A+G modified RNA; right lane: C+U modified RNA. Part of the sequence is displayed in the margins. The complete sequence is given in Materials and Methods.

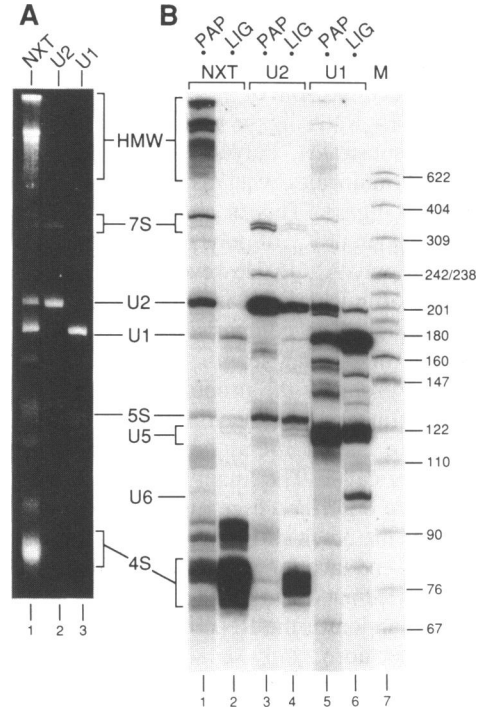

Figure 3. Qualitative comparison of 3′-end labeling with cordycepin by poly(A) polymerase and pCp by T4 RNA ligase **(A)** RNA was purified from HeLa cell nuclear extract (lane 1) and from fractions enriched for U2 (lane 2) and U1 (lane 3) snRNA respectively. After separation on a 10% polyacrylamide gel the RNA was visualized by staining with ethidium bromide. **(B)** RNA preparations (indicated on the top) from (A) were either labeled with cordycepin by poly(A) polymerase (odd numbers) or with pCp by T4 RNA ligase (even numbers) and separated on a 10% polyacrylamide gel. The sizes of DNA-fragments from pBR322 that were generated by digestion with *Hpa*II are indicated at the right-hand side. The assignment of some RNA molecules is given between (A) and (B). Abbreviations used are: PAP (poly(A) polymerase), LIG (T4 RNA ligase), NXT (RNA from HeLa cell nuclear extract), HMW (high molecular weight RNA).

U1 snRNA. The most striking difference is seen with U6 snRNA which is present in the U1 fraction (indicated at the margins). This RNA did not become labeled with poly(A) polymerase. This can be explained since a great portion of U6 snRNA of metazoan cell lines contains at the 3'-end a cyclic 2', 3'-phosphate end group (13) which is resistant to labeling with poly(A) polymerase. It still becomes labeled with T4 RNA ligase. Whether this reflects the minor fraction of U6 snRNA that carries a 2', 3'-diol end group (13) or some other end group is unclear since to our knowledge the 3'-ends of HeLa U6 snRNA have not been investigated.

DISCUSSION

We show that poly(A) polymerase can label 3' termini of RNA by the covalent addition of cordycepin 5'-monophosphate. The reaction product is homogeneous and is suitable for sequence analysis. Incorporation is very efficient and fast, even at low substrate concentrations when poly(A) polymerase is used at high concentrations. Since poly(A) polymerase is still active after several hours of incubation the reaction could probably also be carried out at much lower enzyme concentrations when the incubation time is extended. Previous studies have shown that poly(A) polymerase is more active with higher substrate concentration than the ones used here. The K_m for the RNA primer is about 0.5 μM and the K_m for ATP is approximately 50 μM (4). The volume of the reaction mixture should therefore be kept at a minimum.

A qualitative comparison of 3'-end labeling with poly(A) polymerase and T4 RNA ligase reveals that the two enzymes prefer different substrates. Poly(A) polymerase preferentially labels long RNA molecules whereas short RNA molecules are favored by RNA ligase. This is consistent with the previous finding that the apparent K_m of poly(A) polymerase is higher for oligo(A) than for poly(A) (4). However, *L3pre* RNA, which has a length of only 56 nucleotides, is also efficiently labeled with poly(A) polymerase. The comparison of U1 and U2 snRNA which both are of similar length shows that the sequence composition and probably the pronounced secondary structure of U snRNAs influences the reaction as well. Therefore, the method of choice for a 3'-end labeling reaction should be determined empirically. The main advantage of the 3'-end labeling procedure described here over the reaction with RNA ligase and pCp is the much shorter incubation time and the ease of the method. 3'-end labeling with RNA ligase proceeds slowly and is usually carried out overnight. Such long incubation periods can lead to degradation of the RNA by traces of contaminating nucleases. Furthermore, only a single reaction product is generated with poly(A) polymerase. RNA ligase concomitantly generates circularized and concatemeric RNA molecules, when the RNA is not capped, when pCp is not present in excess, or when circularization is not prevented by the prior removal of the 5' phosphate group (6).

The addition of cordycepin 5'-monophosphate to an RNA 3'-end can not only be used to visualize trace amounts of RNA or to prepare sequencing substrates. RNAs that are blocked at the 3'-end with cordycepin are resistant to most 3' exonucleases and are therefore more stable. Since they cannot be polyadenylated, such RNAs have been used for injecting into *Xenopus* oocytes to investigate the role of regulated polyadenylation (14).

Another application for recombinant poly(A) polymerase is the polyadenylation *in vitro* of RNA molecules that lack a poly(A) tail. This reaction is carried out under the same conditions as described above except that cordycepin 5'-triphosphate is replaced by labeled or unlabeled ATP. The extent of adenylation can be tested in time course experiments and can be controlled by the concentration of ATP that is included in the reaction. With this method cDNA synthesis of bacterial mRNA, viral RNA (15), or histone mRNA could be facilitated by polyadenylation of these RNAs prior to hybridization to oligo dT and reverse transcription.

Messenger RNAs with long poly(A) tails are translated more efficiently in reticulocyte lysates (16). Long tracts of poly(A) could therefore be added to selected mRNAs to increase the yields of translation product.

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