A chemical method to enrich RNA by molecules having 5'-terminal triphosphate groups

P.M.Chumakov*, M.A.Grachev**, S.V.Netesov⁺ and I.N.Shatsky[†]

⁺All-Union Scientific-Research Institute of Molecular Biology, Novosibirsk, 90, USSR

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ABSTRACT

A method is proposed to enrich RNA molecules having a 5'-terminal triphosphate group. The method is based upon selective chemical modification of 5'-triphosphate groups by an antigen-containing amine followed by affinity chromatography on an absorbent loaded with antibodies specific to this antigen. A purification factor up to 37 may be achieved.

INTRODUCTION

Recently Grachev *et al.* (1) have described a method of selective chemical modification of RNA at a 5'-triphosphate group which made it possible to attach various amines to this position of the RNA.

It is known that free 5'-triphosphate groups are present in pro-mRNAs. The parts of pro-mRNAs which contain these groups are usually excised in the course of processing pro-mRNAs into mature mRNAs. The mechanisms of this processing are now being extensively studied. For these studies it is necessary to have efficient methods of separation of 5'-triphosphate-containing RNAs from those which do not contain these groups. Of particular interest is also the hybridization of 5'-triphosphate-containing RNAs with the corresponding fragments of DNA as a means of finding sites of transcription initiation in long DNAs.

The existing techniques for the enrichment of 5'-triphosphate-containing RNAs based upon their increased affinity towards hydroxylapatite (2,3) is not efficient enough.

The present communication describes a method of enrichment of 5'-triphosphate-containing RNA based upon the selective chemical modification of 5'-triphosphate groups by p-amino-

phenyl-O- β -D-lactoside followed by affinity chromatography on an absorbent containing immobilized antibodies against p-aminophenyllactoside (4).

MATERIALS AND METHODS

The following reagents were used : DEAE-cellulose DE-52 of Whatman, England; MES (2-morpholinoethansulfonic acid) and HEPES (N-2'-hydroxyethylpiperasine-2-ethansulphonic acid) buffers of Serva, FRG; labelled nucleoside-5'-triphosphates from Amersham, England. T7 DNA was isolated from T7 phage obtained from a pilot plant of the Novosibirsk Institute of Organic Chemistry. The BsuI-promoter fragment of T7 DNA (5) was kindly given by Dr.E.F.Zaychikov. RNA-polymerase of E.coli was kindly given by Mr.V.Khomov.

CME-carbodiimide (N-cyclohexyl, N'- β -(4-methylmorpholinium)ethylcarbodiimide p-toluenesulfonate) was synthesized according to (6) in the Technological Laboratory of the Novosibirsk State University and additionally purified as described in (1). Antibodies against p-aminophenyllactoside were obtained and purified as described in (4). Immobilization of these antibodies on cyanogen bromide-activated Sepharose (Pharmacia, Sweden) was performed according to the instruction of manufacturer. About 80% of the protein was bound to the adsorbent; activity of the antibodies was retained to an extent of 15%.

Synthesis of p-aminophenyl-O- β -D-lactoside was performed according to (7) with minor modifications. The p-aminophenyllactoside obtained was three times recrystallized from 75% aqueous ethanol and dried in vacuo over CaCl_2 . Colourless needles, m.p. $240-242^\circ$, $\alpha_{25}^D = 23,5^\circ$ (ref.(7): m.p. 240° , $\alpha_{25}^D = 23^\circ$).

Synthesis of the ATP- γ -amidate - a derivative of p-aminophenyllactoside. This γ -amidate (abbreviation LAC-ATP) was synthesized according to the general method (8) by condensation of ATP with p-aminophenyllactoside in an aqueous medium at pH 5.7 in the presence of CME-carbodiimide. The reaction mixture contained : 100 μl dimethylformamide; 18 mg p-aminophenyllactoside; 200 μl water; 20 μl 1M MES, adjusted with NaOH to pH 5.7; 100 μl 0.01M Na_2ATP ; 23 mg CME-carbodiimide. The mixture was kept for 4 hr at 12°C , diluted with 10 ml of water

and applied to a column of DEAE-cellulose (0.9x15 cm). The column was washed with water followed by 0.01 M triethylammonium bicarbonate (TEAB), and elution was performed with 200 ml of a linear gradient of TEAB (0.01 to 0.45 M) at flow rate 20 ml per hour ; 5 ml fractions were collected. The peaks were identified by their UV-spectra. The fractions containing LAC-ATP were combined and evaporated to dryness a few times with water to remove TEAB. Radioactive LAC-ATP from [^{14}C]-ATP (54.5 mCi/mmol) was similarly obtained.

Acid hydrolysis of LAC-ATP. The 10^{-3}M solution of LAC-ATP was kept during 1 hr at pH 1.5, 40° . Micro-column chromatography of the neutralized and diluted hydrolyzate was performed exactly as in (15). The products of hydrolysis were identified as p-aminophenyl lactoside and ATP by their UV-spectra.

Synthesis of RNA with γ -[^{32}P]-5'-triphosphate terminal groups (γ -[^{32}P]-RNA). The reaction mixture (50 μl) contained: $5 \times 10^{-4}\text{M}$ UTP, CTP, GTP and γ -[^{32}P]-ATP (5 Ci/mmol); 0.05M NaCl; 0.05 M tris-HCl pH 8.1; 0.02 M MgCl_2 ; 10 mM 2-mercaptoethanol; 5 μg of BsuI-promoter fragment of T7 DNA (5); 10 μg of RNA-polymerase. The mixture was kept for 20 min at 37° . After this, 10 μl of 0.2 M EDTA was added, and the mixture was three times extracted with phenol, saturated with acetate buffer pH 5.5 (such extraction also removes DNA (9)). 20 μg of phenol extracted rRNA was added, and RNA was precipitated with ethanol. The precipitate was dissolved and subjected to gel-filtration in order to remove phenol and NTPs. The yield of γ -[^{32}P]-RNA was 75.000 cpm (Cerenkov).

The preparation of [^3H]-RNA degraded by cobra venom endonuclease. The reaction mixture (210 μl) contained : $5 \times 10^{-4}\text{M}$ UTP, GTP, CTP and [^3H]-ATP (2 Ci/mmol); 0.05M tris-HCl pH 8.1; 0.02M MgCl_2 ; 0.01M 2-mercaptoethanol; 0.05M NaCl; 15 μg T7 phage DNA; 20 μg E.coli RNA-polymerase. The mixture was kept for 5 hr at 37° , deproteinized by acidified phenol as described above, and subjected to gel-filtration. The yield of [^3H]-RNA was 2×10^6 cpm. After this, 100 μg of carrier rRNA was added and the mixture was treated with 0.1 units of E.coli alkaline phosphatase (BAPF, Worthington, U.S.A.) in 0.1M tris-HCl pH

8.5 - 0.01M MgCl_2 for 1 hr at 37°C ; the mixture was then extracted with phenol. As a result of such treatment, $[^3\text{H}]\text{-RNA}$ completely loses its 5'-terminal triphosphate groups, as indicated by the complete transformation of ATP added to the reaction mixture into adenosine. After removal of phenol by gel-filtration, $[^3\text{H}]\text{-RNA}$ was subjected to limited degradation by cobra venom endonuclease, which gives products of the general formula pNpN...pN (11); the enzyme was kindly given by Dr. S. K. Vassilenko. The reaction mixture (200 μl) contained dephosphorylated $[^3\text{H}]\text{-RNA}$ with carrier; 0.01M MgCl_2 ; 0.25M NaCl ; 0.05M tris-HCl pH 8.0; 0.12 units of cobra venom endonuclease. After 10 min of incubation at 37°C , the mixture was extracted with phenol three times and precipitated with ethanol. The average length of $[^3\text{H}]\text{-RNA}$ obtained was estimated from the ratio of radioactivities of the products of alkaline hydrolysis (Np and pNp); it appeared to be equal to 50 - 75 nucleotide residues.

Selective modification of $\gamma\text{-}[^{32}\text{P}]\text{-RNA}$ at the 5'-triphosphate residues. The selective modification was performed basically according to ref. (1). The mixture contained 10^4 cpm $\gamma\text{-}[^{32}\text{P}]\text{-RNA}$ (Cerenkov) and 10^6 cpm of degraded $[^3\text{H}]\text{-RNA}$ (dioxane scintillation liquor), and also 50 μg of carrier rRNA in 150 μl of 0.02 M MES pH 5.8, 6.6 mg of CME-carbodiimide was added and the reaction was run for 40 min at 15°C . After this, 300 μl of a mixture of 150 μl dimethylformamide, 150 μl 0.1M HEPES pH 8.2 together with 25.8 mg of p-aminophenyllactoside was added. After 1 min, RNA was precipitated with ethanol in the presence of 0.15 M NaCl , the pellet was collected, dissolved in water and re-precipitated with ethanol. The RNA was then dissolved in 300 μl of 0.05M sodium carbonate-bicarbonate buffer pH 10.5 and incubated for 2 hr at 37°C . In the course of such treatment base residues of RNA, which might have been modified to a small extent by CME-carbodiimide during the treatment at pH 8.2, should become completely unmodified (10). The product was desalted by gel-filtration and precipitated with ethanol. The extent of the selective modification of $\gamma\text{-}[^{32}\text{P}]\text{-RNA}$ at the 5'-triphosphate groups, determined as described in ref. (1), was 22% (on average).

Immunochromatography of LAC-ATP. 0.1 nmole of [^{14}C]-LAC-ATP in 0.02M KH_2PO_4 pH 6.0 - 0.02M NaCl - 1 mM NaN_3 (Buffer A) was applied to a column (0.3x10 cm) with antibodies against p-aminophenyllactoside (4), equilibrated with Buffer A. The column was washed at a rate of 2 ml/hr at first by Buffer A and then with 0.02M lactose in Buffer A. Fractions (0.7 ml) were collected and their radioactivity was measured (Fig.1).

Immunochromatography of selectively modified RNA. The mixture of degraded [^3H]-RNA (5×10^5 cpm) and γ -[^{32}P]-RNA (8×10^3 cpm, Cerenkov) selectively modified at the triphosphate residues as described above was subjected to immunochromatography in Buffer A followed by Buffer A with 0.02M lactose, as described in the previous section, with the exception that at both stages Buffer A was supplemented with tRNA (20 $\mu\text{g}/\text{ml}$) to decrease non-specific absorption. Radioactivity of [^{32}P] was at first determined by Cerenkov effect, and after this [^3H] was counted in dioxane scintillation liquor. The result is shown in Fig.2.

Evaluation of the extent of degradation of γ -[^{32}P]-RNA in the course of selective modification. This was performed by means of gel-electrophoresis. Electrophoresis was run in 7 M urea in an 8% polyacrylamide gel according to ref.(12). An aliquot of RNA subjected to selective chemical modification as described in the previous section was applied. In a parallel lane applied was a HaeIII-digest of single-stranded ϕX174 DNA (12), which consists of fragment of the following lengths: 1342, 1078, 872, 606, 310, 278, 271, 234, 194, 118, 72 nucleotide residues (the digest was kindly given by Dr. V.V.Kravchenko). After the run, the gel was washed with water (2x30 min; washing does not remove RNA as shown by control experiments) and stained with a solution of ethidium bromide. The marker bands were visualized by fluorescence induced by UV-light. After this, the lane which contained selectively modified RNA was cut into 0.5 cm strips and the latter were counted in water [^{32}P] (Cerenkov effect). The results of the electrophoresis are shown in Fig.3.

RESULTS AND DISCUSSION

During the elaboration of the method of enrichment of RNA by molecules with 5'-triphosphate groups, we based our approach on the method of selective modification of RNA at the 5'-triphosphate groups which was described by us earlier (1). According to this method, various amines may be attached selectively to the 5'-triphosphate groups of RNA.

We decided to attach to these residues a haptene - p-aminophenyl lactoside, and to separate the mixture of modified and unmodified RNAs by means of immunochromatography on a column with antibodies against lactose. The same haptene has been employed recently to label the 3'-end of 16S RNA for subsequent electron-microscopic visualization of this group on surface of ribosomes (4).

For model experiments, we at first synthesized a lactose-containing ATP- γ -amidate (LAC-ATP) of following structure:

The synthesis was performed using the general procedure (8) based upon intermediate formation of adenosine-5'-cyclo-trimetaphosphate and its subsequent reaction with p-aminophenyl lactoside. The structure of LAC-ATP is in accord with the following data: (i) the compound has a net charge (-3), as suggested by its mobility in ion-exchange chromatography compared to ATP; (ii) the UV-spectrum of the compound contains maxima characteristic of the UV-spectra of both ATP and p-aminophenyl lactoside; (iii) acid hydrolysis of this compound gives a quantitative yield of ATP; (iv) it is firmly established (1,8,13,15,18), that the method of synthesis employed always gives γ -amidates of NTPs.

Fig.1 shows the result of chromatography of radioactive LAC-ATP on an immunoadsorbent loaded with antibodies against p-aminophenyl lactoside. It is seen that LAC-ATP, taken in an appropriate amount, is completely and firmly bound with the

Figure 1. Immunochromatography of radioactive [^{14}C]-LAC-ATP. The arrow shows the moment when added to the eluent was lactose. ATP in control experiments was completely eluted in fractions 1 to 4.

sorbent and is desorbed selectively by exactly the same buffer supplemented with lactose.

It is important that the immunoabsorbent column may be used many times. For regeneration it is sufficient to wash the column extensively with Buffer A. The average life-time of the column is several months when stored in refrigerator in between experiments.

The next task was to prove the validity of the method with a model mixture of RNAs. The first component of such a mixture was RNA synthesized by means of *E. coli* RNA-polymerase from γ -[^{32}P]-ATP and three non-labelled NTPs on the promoter-containing fragment of T7 DNA as template. This fragment has a length of ca. 1400 b.p.; it was isolated from the *Bsu*I-digest of T7 DNA according to ref.(5). The fragment contains the left-hand terminus of T7 DNA and the promoters A_0 , A_1 , A_2 , A_3 of which two (A_1 and A_2) initiate synthesis of RNAs with 5'-terminal ATP residues having lengths 950 and 700 nucleotide residues (14).

The second component of the model mixture was [^3H]-RNA, synthesized on a T7 DNA template. This RNA was at first dephosphorylated to remove any 5'-triphosphate groups, and subsequently degraded by means of cobra venom endonuclease to fragments of structure pNpN...pN (11), the lengths of which were 75 - 100 nucleotide residues. This degraded RNA as a model of RNA,

having no 5'-triphosphate groups, gave the possibility of a stringent check because 5'-phosphate residues are the most reactive groups of all the possible side targets of the selective modification procedure.

It is known that internucleotide phosphates are many times less reactive in activation by various reagents compared with the terminal ones, and that the activated 3'-phosphates mainly react intramolecularly, yielding 2',3'-cyclophosphates (17), and therefore they have little chance of being modified by the amine. At the same time, the length of our model RNA of the structure pNpN...pN was much smaller than that of the natural mature RNA isolated from living cells, and this fact also increases the strictness of our evaluation.

Fig.2 shows the result of immunochromatography of the model mixture of RNAs after the selective modification by p-aminophenyl lactoside. It is seen that almost all $[^3\text{H}]$ -RNA is eluted from the column by buffer containing no lactose, whereas 25% of γ - $[^{32}\text{P}]$ -RNA is eluted only after the buffer is supplemented by lactose. The extent of the enrichment of γ - $[^{32}\text{P}]$ -RNA in different experiments varies between 25 and 37.

Figure 2. Immunochromatography of the model mixture of γ - $[^{32}\text{P}]$ -RNA and $[^3\text{H}]$ -RNA after selective chemical modification of the 5'-triphosphate groups with p-aminophenyl lactoside. The arrow shows the moment of lactose addition to eluent.

—●—●— radioactivity of $[^{32}\text{P}]$, Cerenkov (cpm); right ordinate.
 —○—○— radioactivity of $[^3\text{H}]$, dioxane scintillation liquor (cpm); left ordinate.

This result is much better than that obtained by the existing method, based upon greater affinity of the triphosphate group to hydroxylapatite (2,3); the latter gives extents of enrichment equal to only 6 even after two consecutive rechromatographies. Moreover, the chemical method proposed is very reproducible, which is not the case with the hydroxylapatite method.

A disadvantage of the method is the low yield of chemical modification (20-25%). This low yield is due to the low basicity of the amine - p-aminophenyl lactoside.

The yield could be increased if the condensation was performed at pH 5.7 directly in the presence of the amine. However, it was evident from theory and it was proved experimentally that the selectivity decreases considerably in such a single-step procedure. The selectivity is determined by the fact that the cyclic trimetaphosphate which is formed in the reaction of CME-carbodiimide with the 5'-triphosphate residue is the only relatively long-living active phosphorylating intermediate ($\tau_{1/2} = 7.5$ min at 15° (15)), which is accumulated in large concentration (yield up to 75%). All the other phosphate residues give only very unstable phosphorylating derivatives, the steady-state concentration of which is very small. After the reaction the mixture is brought to pH 8.0 by addition of HEPES (see "MATERIALS AND METHODS"), all the unstable derivatives are hydrolyzed. When the amine is added after a while, it reacts only with the remaining 5'-trimetaphosphate. However, if the activation is performed directly in the presence of the amine (which is possible in case of weakly-basic amines, like aniline, p-aminophenyl lactoside, etc.), all the active phosphorylating intermediates have the possibility of reacting with the amine giving amides, i.e., the selectivity drops dramatically.

To increase the yield, it might be possible to take a more basic amine in order to change favourably the ratio of the rates of hydrolysis and aminolysis of the trimetaphosphate. However, our attempts to synthesize and to employ a derivative of lactose having a basic amino group up to this time have failed.

We have mentioned earlier (1), that treatment of RNA with

CME-carbodiimide results in some degradation of RNA, which is presumably due to the formation of triesters from the activated internucleotide phosphates (cf. ref.(16)).

Of some relevance to this is the fact that very rapid degradation and isomerization of RNA takes place when it is treated with different condensing reagents in non-aqueous media (16).

It was found that treatment of RNA with CME-carbodiimide in an aqueous solution under the above mentioned conditions of selective modification of 5'-triphosphate groups does in fact result in degradation, but to a small extent. Particularly,

Figure 3. Gel-electrophoresis of the model mixture of RNAs after selective modification. The gel was cut into 0.5 cm pieces and counted for [³²P] (Gerenkov). Marked are positions of HaeIII-fragments of ϕ X174 single-stranded DNA, the figures correspond to lengths (nucleotide residues) of these fragments. Shown are also positions of the starting γ -[³²P]-RNA-950 and γ -[³²P]-RNA-700.

γ -[^{32}P]-RNA consisting of molecules, having lengths 950 and 700 nucleotide residues, after the modification procedure yielded products having an average length of some 100 nucleotide residues with a wide distribution of molecular weights, as revealed by gel-electrophoresis (see Fig.3).

It must be especially mentioned, that all the procedures were performed under the conditions which decreases to a minimum the possibility of contamination by nucleases (all the solutions were treated by bentonite). Hence, it is most probable that the degradation is in fact due to the action of CME-carbodiimide.

It is noteworthy, that this extent of degradation is not an obstacle for the use of enriched RNA for many purposes like, e.g., various studies based upon its hybridization with DNA.

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* Institute of Molecular Biology, USSR Academy of Sciences, Moscow

** Novosibirsk Institute of Organic Chemistry, Siberian Branch of the Academy of Sciences of the USSR, Novosibirsk

+ To whom requests for reprints should be sent

† Belozersky Interfaculty Laboratory of Molecular Biology and Bioorganic Chemistry of the Lomonosov Moscow State University, Moscow

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