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In vitro Transcription and Purification of RNAs of Different Size

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Abstract: In vitro run-off transcription from a double-stranded DNA template by T7 RNA polymerase is an elegant way to obtain highly pure and uniform RNA oligonucleotides of lengths ranging from about 15 to several thousand nucleotides. Here we describe the different strategies applied and optimized in our laboratory to enzymatically synthesize RNAs as necessary when working at the interface of bioinorganic chemistry, coordination chemistry, RNA biochemistry and structural biology.

Keywords: Plasmids · RNA · T7 RNA polymerase · Transcription

1. Introduction

Interdisciplinary research is gaining increasingly in importance and thus the borders between ***chemistry*** and other research fields are vanishing more and more. In our laboratory we work at the interface between ***structural biology***, ***bioinorganic chemistry***, and ***inorganic chemistry*** of RNA [1–5]. In this short review we provide a general overview concerning different methods of RNA synthesis that we have optimized according to our requirements.

The RNA oligonucleotides we are studying range from 7 to 2500 nucleotides (nt) in length. RNAs are commercially available only to a limited extent: Although chemical synthesis up to a length of about 80 nucleotides is possible, with increasing length (>20 nt), yields decrease rapidly and costs become prohibitive. A good alternative is to make the RNA oneself by *in vitro* transcription with T7 polymerase, which is a standard and well-described procedure applied in numerous biochemical laboratories around the world [6]. Our research focuses on the interaction of metal ions with ribozymes, *i.e.* catalytic RNAs, and includes structure determination [3][4] of these RNAs by both NMR and X-ray crystallography (see also [1]) [2][5][7][8]. Consequently, we have special requirements regarding yield and uniformity in length, as well as buffers and metal ions employed during transcription and purification. We have therefore set up and optimized an efficient system of RNA transcription and purification to synthesize RNAs according to our needs.

2. General Considerations and Chemical Synthesis of RNA

RNA is commonly prepared by one of the three following ways:

(i) by direct isolation and purification from the biological source, which are not further described in this paper;
(ii) by chemical synthesis on a nucleic acid synthesizer;
(iii) by *in vitro* run-off transcription from dsDNA (double-stranded DNA, see List of Abbreviations below) using T7 polymerase.

The chemical synthesis of RNA sequences has been described in detail [9] and is usually carried out by companies or in specialized laboratories [10]. Standardized chemical synthesis of RNA is one of the significant achievements in RNA (bio)chemistry of the past few years and is the method of choice in the following cases:

(i) if short sequences (<15 nt) are needed, which are usually difficult to obtain in good yield and purity by transcription;
(ii) if a specific sequence at the 5′-end of the RNA is required that cannot be obtained by *in vitro* transcription (see also below);
(iii) if non-standard nucleotide derivatives have to be incorporated site-specifically into the sequence. Such modifications are for example heavy-atom derivatives, *e.g.* 5-bromo- and 5-iodouridine [11], or selenium-modified ribonucleotides [12][13], all of which are needed for X-ray crystallography. Other derivatives serve as fluorescent markers [14] or are used for NAIM [15][16] and NAIS studies [17].

Major limitations of the chemical synthesis of RNA are the length of the sequence, the amount of RNA which can be produced, and the price. As every nucleotide is introduced with an efficiency of about 97%, yields decrease dramatically for RNA sequences longer than 30 nt. Other encountered disadvantages of commercially available chemically synthesized RNA are impurities such as capped aborts and incompletely deprotected sequences, in addition to prohibitive prices, if large amounts are needed.

For many applications it is therefore useful and necessary to synthesize the RNA by *in vitro* transcription. In the following sections, we will shortly describe methods for the synthesis of RNA taking into account its future application, as well

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as sequence, purity, and length uniformity of the target RNA.

3. *In vitro* Transcription

To bypass the limitations of chemical synthesis indicated above, the enzymatic RNA synthesis by RNA polymerases is a good alternative. Bacteriophage RNA polymerases, such as T7, are exceptionally active in *in vitro* transcription and have the advantage of initiating transcription at a single site and to terminate quite precisely [6]. Like many enzymes, T7 can be obtained commercially. However, as soon as milligram amounts of RNA are needed, as is the case for any structural investigation, T7 needs to be applied in high concentrations, which again becomes financially prohibitive. We therefore efficiently express T7 in our laboratory and have optimized its purification.

3.1. *T7 RNA polymerase*

Bacteriophage T7 RNA polymerase is a 99 kDa enzyme composed of a single polypeptide chain. This protein recognizes a specific dsDNA sequence of 17 nt in length, the so-called promoter sequence (Fig. 1). Transcription, *i.e.* the sequence-specific generation of a RNA chain from NTPs, is initiated after the TATA box at position +1 (Fig. 1). T7 thereby melts the dsDNA template riding along the template strand, which encodes the wanted RNA sequence [6]. In contrast to DNA polymerases, addition of an oligonucleotide-primer is not necessary. Incorporation of the first nucleotides is called the initiation phase, which is characterized by abortive cycling, a process leading to the production of short RNA sequences of typically 2–6 nt in length [18]. After a conformational change of the enzyme, the elongation complex in form of a RNA-primed DNA template is formed, which now completes transcription of the entire sequence in a highly processive and accurate manner [19].

In our group, we express T7 RNA polymerase from the *E. coli* strain *BL21(DE3)* [20] containing the expression plasmid pBH112. The plasmid encodes (i) for T7, to which a 6x His tag is added, and (ii) for an ampicillin resistance. Typically, two 500 ml cell cultures are grown in shaking flasks at 37 °C in LB medium containing ampicillin. As soon as an optical density of 0.6 at 600 nm is reached, IPTG is added to a final concentration of 0.4 mM to induce expression of T7. Four hours after induction, the cells are harvested by centrifugation at 6000 g for 4 min leading to pellets, which can be stored at –80 °C. Cell lysis is achieved by resuspension in 20 mM Tris-HCl buffer (pH 8.0), 500 mM NaCl, 1% deoxycholate, 0.05 mM PMSF and subsequent sonification. After centrifugation, the viscous brown supernatant is applied to a Ni^{2+}-NTA column and washed with 10 mM imidazole in 20 mM Tris-HCl pH 8.0, 500 mM NaCl. The bound T7 RNA polymerase is then eluted with 100 mM imidazole in the same buffer and concentrated by centrifugation through filter devices.

In order to obtain highly pure and active T7 polymerase in good yields we added the following steps to the general protocol: The polymerase is further purified by chromatography on a cation exchange column that has been equilibrated with 30 mM HEPES buffer (pH 7.5), 100 mM potassium glutamate, 0.25 mM EDTA, 0.05 % Tween-20, 1 mM DTT. The bound protein is eluted by application of a 250 ml gradient of NaCl ranging from 0 to 1 M. The peak fractions eluting near 200 mM NaCl are pooled and concentrated in filter devices to a volume of typically 6 ml. After addition of an equal volume of glycerol, the polymerase is stored at –20 °C for immediate use or flash frozen in liquid nitrogen for long-term storage at –80 °C. This procedure yields at least 50 mg of pure and highly active T7 RNA polymerase.

3.2. *General Transcription Requirements*

Before considering the specific requirements and conditions for transcription of RNA of different lengths (see Sections 3.3 and 3.4) we first summarize the important points to be considered in all cases.

As mentioned above, the promoter region needs to be double-stranded to bind T7. The following DNA template sequence can be single-stranded. Thus, the so-called template strand contains (from 3′ to 5′) the promoter sequence followed by the coding sequence ('template'). In contrast, the top strand can be much shorter encompassing only the complementary promoter sequence plus some additional Gs. In our experience, fully dsDNA oligonucleotides usually give the best yields. However, there are no rules about which variation is the best: On the one side, a fully dsDNA template often increases transcription yield, but on the other side a short top strand can be universally used for any template strand.

The DNA template can be obtained from different sources: (i) From two chemically synthesized strands, (ii) by amplification from a tiny amount of dsDNA by PCR, or (iii) from a linearized plasmid [21].

The first six nucleotides at positions +1 to +6 have the greatest impact on transcription initiation and the subsequent structural change of T7 to the elongation complex; hence, these nucleotides determine the yield of the target RNA to a large amount. A guanosine at position +1 is by far the best starter, but in principle any one of the four nucleotides work [22]. Many possible combinations of G, A, C, and U have been tested for the first six nucleotides [22]. Some excellent starting sequences are: GGGAGA, GGGAUC, GGCAAC, or GGCGCU.

Once a DNA template is at hand, standard conditions for transcription are as follows: 40 mM Tris-HCl (pH 7.5), 40 mM DTT, 2 mM spermidine, 0.01% Triton-X 100, 5 mM NTP each, 10–50 mM $MgCl_2$,

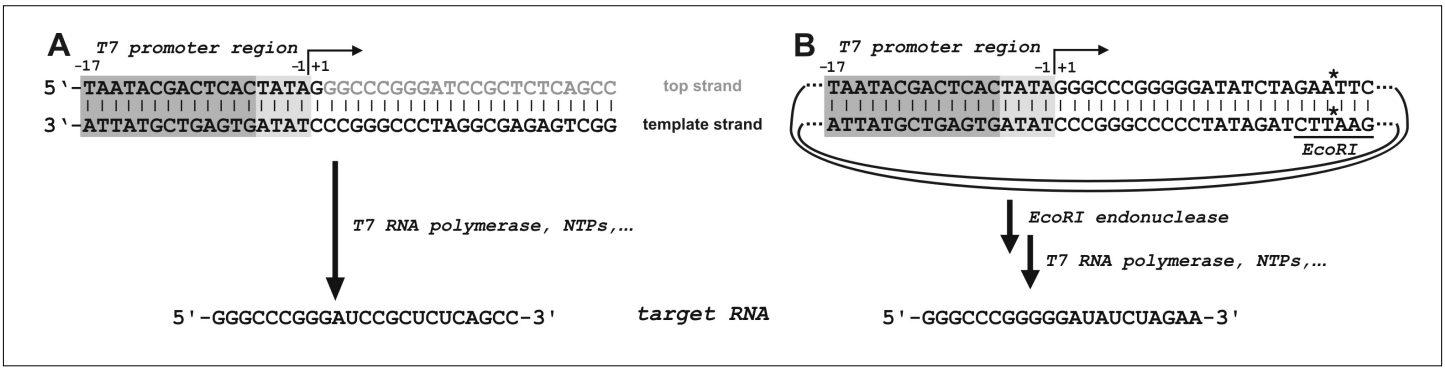

Fig. 1. Transcription of RNA from a dsDNA template. The T7 promoter region excluding the TATA box (position –4 to –1) is marked by a grey box. Transcription starts at position +1, as indicated by an arrow. (A) Short RNA sequences are transcribed from a synthetic partially ss or ds DNA template. The top strand oligonucleotide must contain the nucleotides –17 to +1. The complementary template sequence (shown in grey letters) is not absolutely required. The template strand encompasses the T7 promoter region plus the coding template sequence. (B) Larger RNA sequences are often transcribed from a linearized plasmid. The endonuclease cleavage site, for EcoRI in this case, is indicated by an asterisk.

1.2 μM template or 5–10 μg linearized plasmid/ml transcription solution.

T7 RNA polymerase is always added last in an amount depending on the quality and concentration of the batch and mixed very gently. The DTT solution must be prepared freshly, the spermidine and NTP stock solutions can be stored at –20 °C for several months.

The total volume of a transcription reaction is scalable from a few microliters up to many milliliters and depends mainly on the subsequent application of the RNA. Experience shows that the yield increases when the transcription reaction is carried out in small aliquots, *e.g.* 1 ml each. After mixing, the transcription reaction is incubated at 37 °C overnight (short constructs) or for 2–4 h (very long constructs).

Variables of the standard conditions include the concentrations of NTPs (1–10 mM), $MgCl_2$ (10–50 mM), and the dsDNA template. The concentration range for short oligonucleotide-templates is 0.6–2 μM while for plasmid-substrates the concentration varies between 5–15 μg/ml. Addition of BSA (100 μg/ml) or PEG 8000 (80 mg/ml) sometimes also increases the yield. Our experience shows that the addition of 5 mM GMP can increase transcription efficiency significantly. GMP is only inserted as the starting nucleotide at the 5'-end, but as the initiation of transcription is an important yield-determining factor, increasing the total guanosine concentration can be a good choice. Obviously, the addition of GMP will lead to a mixture of triphosphate and monophosphate 5'-termini, which are well detectable by NMR [4]. This (mostly irrelevant) inhomogeneity can be overcome by subsequent treatment with calf intestinal alkaline phosphatase (CIAP) resulting in homogeneous 5'-hydroxyl ends.

In most cases, standard reaction conditions as given above will yield sufficient amounts of RNA for biochemical studies. However, for NMR and X-ray applications larger amounts of RNA are needed, and thus the reaction conditions have to be optimized for each new template, which is usually done by small-scale (50 μl) reactions.

3.3. *Transcription of Short RNAs (10–50 nt)*

In vitro transcription of short RNAs, *i.e.* about 10–50 nt in length, is usually done from chemically synthesized dsDNA templates. Including the promoter region (Fig. 1), the DNA template will have a length of up to 70 nt, and can thus be readily purchased. Prior to use, the DNA has to be purified by PAGE, followed by electroelution (Schleicher&Schuell) and ethanol precipitation.

To obtain milligram quantities of RNA, usually large-scale transcriptions (10 ml) are run overnight (although 6–8 h is mostly sufficient). Evidently, for large-scale transcription high concentrations of NTPs, dsDNA template, and T7 are needed.

T7 has the peculiarity to run off the DNA template strand and sometimes unspecifically add up to five or six nucleotides at the 3′-end of the RNA transcripts. To separate these so-called N+1, N+2, *etc*. run-off transcripts, as well as abortive shorter sequences from the target RNA, high resolution PAGE is the method of choice. Under large-scale conditions as described here, a white precipitate composed of $Mg_2P_2O_7$ (magnesium pyrophosphate, *i.e.* diphosphate) is often formed during the reaction. This precipitate should be removed by centrifugation prior to PAGE. Alternatively pyrophosphatase can be added during transcription efficiently hydrolyzing the precipitate. The RNA is purified by PAGE followed by electroelution and ethanol precipitation. As a last step, the target RNA is exchanged into water or a suitable buffer by vivaspin concentration (Vivaspin concentrator from Vivascience). It should be mentioned here that the often applied Tris buffer should be avoided when working with metal ions, as this molecule is a good ligand for many M^{n+} ions [23].

3.4. *Transcription of Long RNAs (>50 nt)*

As the length of chemically synthesized DNA reaches its practical limit at around 80 nt, transcription of RNA sequences >50 nt is usually done using plasmid DNA as a template. The sequence of interest is obtained by common PCR or by colony PCR directly from the organism and is then cloned downstream of a T7 promoter into a multicopy plasmid by standard cloning procedures [24]. A variety of transcription vectors containing a T7 promoter sequence leading a multiple cloning site is commercially available. We usually follow a slightly different strategy and place the T7 promoter by PCR directly upstream of the coding DNA sequence. This strategy prevents the insertion of unwanted 5′-terminal nucleotides. However, it leads to some restrictions on the first six nucleotides of the transcript as discussed above.

T7 moves along the dsDNA template as long as possible, *i.e.* there is no stop signal. Thus, the plasmid has to be cleaved sequence specifically by a restriction endonuclease at the 3′-end of the coding sequence. Complete restriction is essential as small percentages of uncut plasmid will reduce the yield dramatically by production of very long runarounds that use up most of the NTPs present in solution. After complete digestion with the restriction enzyme, the cleaved plasmid is purified with the best suitable purification kit.

Standard conditions are employed for transcription as stated above. Generally 5–10 μg cleaved plasmid per ml transcription solution are used. The transcription time usually is between 2–4 h and gets shorter the longer the RNA sequences are (because of increasing sensitivity to degradation).

Isolation of the RNA is performed as described above for shorter RNAs. If very long RNA sequences (> 200 nt) are to be transcribed, a better recovery from the polyacrylamide gel might be achieved by *crush and soak*. Thereby, the gel pieces containing the RNA are squeezed through a syringe, frozen, and eluted twice with at least double the volume of elution buffer (10 mM MOPS (pH 6–7), 1mM EDTA, 250 mM NaCl) at 4 °C.

The transcription using plasmid DNA has several advantages: The most important one is that there is no limitation in length regarding the coding DNA and thus also the target RNA sequence. The plasmid can be amplified in bacteria in a cost-saving manner and purified by standard procedures.

Naturally also this method leads to N+1, N+2, *etc*. fragments, as well as sometimes even to inhomogeneous 5′-ends [25][26]. Due to the small difference in length of these run-off transcripts compared to the overall length, it is often difficult to separate these longer transcripts from the target RNA by PAGE. This often causes problems when highly homogeneous RNA is needed, *e.g.* for X-ray crystallography. An elegant way to obtain a homogeneous transcript is shortly described in the following Section.

4. RNAs with Uniform 5′- and 3′-Ends

To circumvent inhomogeneous ends of the target RNA, ribozyme sequences can be placed 5′ and 3′ to the desired RNA (Fig. 2) [27–30]. The two flanking ribozymes are co-transcribed yielding one long RNA with the target sequence in the center. Subsequent to transcription, both ribozymes cleave at a unique location defined by a specific sequence.

To obtain a homogenous 5′-OH end, a *cis*-acting, *i.e.* self-cleaving, hammerhead (HH) ribozyme is a good choice. The HH cleaves 5′ of a ten nucleotide sequence, which it recognizes through Watson-Crick base pairing with nucleotides being part of the HH. Thus, for each target RNA, the 47-nt long HH sequence has to be individually modified and introduced between the T7 promoter region and the target RNA by PCR [30].

The usage of a *cis*-HDV (hepatitis delta virus) ribozyme sequence will lead to a homogeneous 3′-end. The HDV-ribozyme cleaves 3′ after the last base of the target RNA sequence, which has to be any base other than G [31]. Alternatively, if a 3′-G

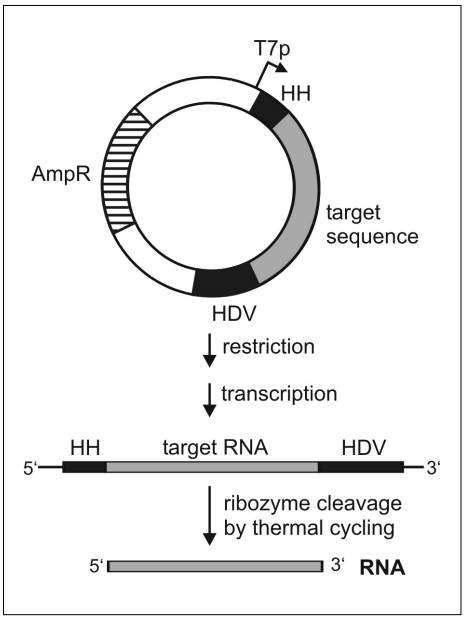

Fig. 2. Schematic view of the preparation of a target RNA with homogeneous ends. The plasmid derived from the 5′-HH/3′-HDV ribozyme vector pRZ [30] contains the target coding sequence flanked by two self-cleaving ribozymes. Transcription leads to the target RNA neighbored by the HH and the HDV ribozymes. Thermal cycling quantitatively yields the pure target RNA (Fig. 3). AmpR, ampicillin resistance; HDV, hepatitis delta virus ribozyme; HH, hammerhead ribozyme; T7p, T7 promoter region.

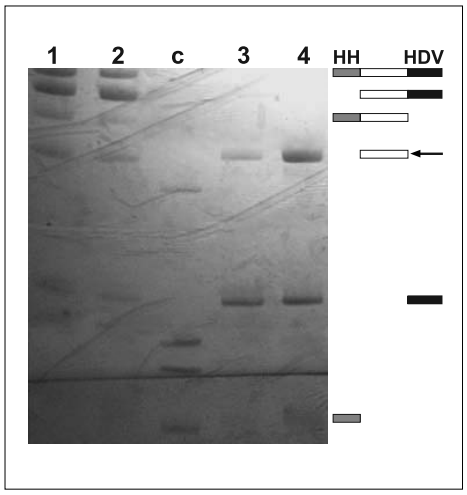

Fig. 3. Analytical polyacrylamide-gel (8%) of transcription trials from the HH-targetRNA-HDV plasmid template as seen by UV shadowing. On the right side, the different species are indicated. Lanes 1 and 2 show the distribution of the different species of the RNA right after transcription. Lanes 3 and 4 show the same sample after adjusting the Mg^{2+} concentration to 40 mM and subsequent three rounds of thermal cycling (see also text). The HH and HDV ribozymes are now almost fully cleaved off leading to large increase in intensity of the target RNA band (arrow). In lanes 1 and 3 [$MgCl_2$] equals 20 mM and in 2 and 4 [$MgCl_2$] equals 30 mM during transcription. Lane c contains markers of different lengths.

is essential, one can also process the target RNA with the *trans*-cleaving, *i.e.* acting on a substrate, VS-ribozyme (*Neurospora* varkud satellite), which cuts after any base other than C [32–35], or by applying a DNAzyme [36]. Thus any desired sequence can be obtained.

General protocols to produce transcripts with homogeneous ends using the HH/HDV- [30] VS/HDV- ribozymes [29], and DNAzymes [36] were published recently.

In our laboratory, we usually apply the HH/HDV approach. The dsDNA template encompassing the T7 promoter, the HH, the target, and the HDV sequence, all flanked by two different restriction sites, is obtained by multistep PCR, and cloned into a plasmid (*e.g.* the pRZ-vector) (Fig. 2) [30]. The plasmid is amplified according to standard procedures and linearized as discussed above. The three RNA oligonucleotides resulting from T7 run-off transcription, done under standard conditions, can then be separated easily by PAGE.

Sometimes incomplete cleavage of the flanking ribozymes occurs. This can be overcome by adjusting the $MgCl_2$ concentration to 40 mM and performing additional thermal cycling after transcription [30]: The solution is heated 1 min at 72 °C, 5 min at 65 °C and 10 min at 37 °C. This process is repeated three times and significantly increases the yield (Fig. 3).

5. Further Purification Methods

As described above, after transcription the RNA is purified by denaturing PAGE to separate the target RNA from the longer N+1, *etc*. transcripts, the DNA strands, T7, as well as the remaining NTPs and other chemical substances of the transcription mixture. For most biochemical applications it is sufficient to redissolve the RNA pellet (after elution and ethanol precipitation) in the desired buffer, *e.g.* 10 mM MOPS (pH 6.5), 10 μM EDTA [7].

For structural studies it is necessary to exchange the solvent more rigorously either by repeated spinning through centrifugal devices or by dialysis. Sometimes it is difficult to completely remove all contaminations from the RNA sample as is evident from NMR spectra. In such a case the sample has to be purified additionally on a 1.2 m long gel filtration column *via* FPLC. Separation by gel filtration is carried out under native conditions, *i.e.* the secondary (and tertiary) structure of RNA is not melted. The same is true for purification on agarose gels, as well as under most conditions for HPLC, two methods which are not further described here. Purification using native conditions has a further advantage: RNA sequences that form hairpins, can also dimerize to form a long duplex. Depending on the sequence, both forms can be present in solution. Under denaturing conditions (*i.e.* in the presence of urea or formaldehyde), the molecules form only a single population of ssRNA molecules, which would reestablish the equilibrium hairpin/duplex after work-up. In contrast, when separated under native conditions, *e.g.* by gel filtration, based on their different size, the isolated hairpin population will not dimerize to duplices as long as the molecules are not heated up to form single strands.

6. Incorporation of Modified Nucleotides into RNA

T7 polymerase recognizes a large variety of chemically modified NTPs and incorporates them into the growing RNA strand (although not always as efficiently as the natural nucleotides). Naturally, these modified nucleobases are incorporated throughout the RNA. For many biochemical purposes (*e.g.* NAIS, NAIM, and UV cross-linking studies), a modified NTP is added in addition to the four common NTPs during transcription. This leads to a statistical incorporation of the modification into the RNA transcripts. The only position where a modified nucleotide can be incorporated site-specifically by transcription is the 5'-G by adding a GMP derivative, *e.g.* α-thio-5'-GMP, to the transcription solution. As GTP is additionally present in every transcription solution, incorporation of the modified GMP at the 5'-end occurs only statistically (see also Section 3.2). To introduce a point mutation into a RNA chain, the oligonucleotide has to be synthesized chemically. If longer RNA sequences are needed, it is possible to ligate this sequence harboring the mutation to another RNA oligonucleotide by a DNA/RNA ligase [37].

Usually, T7 incorporates modified nucleotides as one would expect, *e.g.* 2′-methoxy-ATP is incorporated like an A, or 6-thio-GTP as a G. However, some exceptions apply, one of which is that 2-aminopurine is incorporated in place of A, rather than G. When incorporating thiophosphate derivatives, it is important to know that T7 will selectively produce the R_P diastereomer (and not the S_P: nucleic acids have two diastereotope non-bridging oxygen atoms at each phosphate group, the R_P points towards the major and the S_P towards the minor groove).

For the purpose of NMR structure determination of RNAs, as done in our laboratory, ^{13}C and/or ^{15}N labeled oligonucleotides are needed [1]. NTPs can be purchased either uniformly ^{13}C,^{15}N labeled, or many variations thereof. Even partially or fully deuterated NTPs are available [38]. The availability of such labeled NTPs and the subsequent incorporation into RNA oligonucleotides by *in vitro* transcription as

described above enables us to structurally characterize and investigate larger RNAs (*i.e.* >20 nt) by NMR [1].

7. Summary

In this review we have summarized the various strategies to synthesize and purify RNAs of different lengths according to optimized procedures in our laboratory. *In vitro* run-off transcription by T7 RNA polymerase from a dsDNA template is a very useful method to synthesize single stranded RNA oligonucleotides in large quantities and homogeneous conformation. However, this is not an easy task. In biochemical applications, like activity assays of ribozymes, often only about 80% of a correctly folded and active population is achieved. Such a mixture is unacceptable for our NMR and X-ray studies: NMR spectra become uninterpretable and chances to crystallize RNA diminish rapidly. It follows that purification and exchange of the RNA into the desired solvent is a very important process for the research in our laboratory.

In vitro transcription is a well-established method to synthesize RNA, but experience shows that every new RNA sequence behaves slightly differently. This is not only true for the transcription yield but also for the subsequent purification. It is therefore necessary to treat every new transcription like a new chemical reaction and optimize it accordingly – a process that can be very tedious. Because of the numerous small problems associated with RNA synthesis in general, as well as many new functions of natural RNA molecules discovered almost monthly, it is not surprising that several papers are published every year that address and improve *in vitro* transcription of RNA.

Abbreviations

BSA, bovine serum albumin; CIAP, calf intestinal alkaline phosphatase; DNAzyme, catalytic DNA; dsDNA, double-stranded deoxyribonucleic acid; DTT, dithiothreitol; EDTA, ethylene-N,N,N',N'-diaminetetraacetate; FPLC, fast protein liquid chromatography; GMP, guanosine 5′-monophosphate; HDV, hepatitis delta virus ribozyme; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HH, hammerhead ribozyme; HPLC, high pressure liquid chromatography; IPTG, isopropyl-thio-β-D-galactoside; LB, Luria-Bertani; MOPS, 3-N-morpholino propansulfonic acid; NAIM, nucleotide analog interference mapping; NAIS, nucleotide analog interference suppression; nt, nucleotide; NTA, nitrilotriacetat; NTP, nucleoside 5′-triphosphate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PEG, polyethylene glycol; PMSF, phenylmethanesulfonyl fluoride; ssDNA/RNA, single-stranded DNA/RNA; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; VS, Neurospora varkud satellite ribozyme.

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