

A one-step method for *in vitro* production of tRNA transcripts

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Received May 16, 2002; Revised and Accepted August 15, 2002

ABSTRACT

Sequencing of a large number of microbial genomes has led to the discovery of new enzymes involved in tRNA biosynthesis and tRNA function. Preparation of a great variety of RNA molecules is, therefore, of major interest for biochemical characterization of these proteins. We describe a fast, cost-effective and efficient method for *in vitro* production of tRNA transcripts. T7 RNA polymerase requires a double-stranded DNA promoter in order to initiate transcription; however, elongation does not require a double-stranded DNA template. A partially double-stranded transcription template formed by annealing of a short oligonucleotide, complementary to the T7 promoter, to a larger oligonucleotide is shown to be a good substrate for *in vitro* transcription. This method allows rapid production of a variety of tRNA transcripts which can be aminoacylated well. This eliminates the need for cloning of tRNA genes, large-scale plasmid preparation and enzymatic digestion.

INTRODUCTION

T7 RNA polymerase has been widely used to produce tRNA transcripts *in vitro*. *In vitro* synthesis of tRNA has allowed biochemical characterization of a large number of enzymes and pathways, such as maturation and modification of tRNAs (1), tRNA aminoacylation (2), tRNA export from the nucleus (3) and translation in general (4). A standard method for *in vitro* transcription is based on recognition of the specific promoter sequence by T7 RNA polymerase and subsequent transcription of tRNA genes encoded in double-stranded plasmid DNA. Synthesis of the tRNA 3′-terminal CCA sequence is accomplished by run-off transcription with T7 RNA polymerase using a template generated by enzymatic digestion (typically *Bst*NI) (5).

This method has been proven to be successful, but it occasionally causes addition or omission of nucleotides at the 3′ end, which impairs the ability of such tRNAs to be aminoacylated (6,7). Another drawback of the method arises from the fact that RNAs can be successfully transcribed *in vitro* only if the first base is a purine (8,9). If the first base of the RNA is a cytosine or a uracil, the transcription start site is often altered and erroneous nucleotides are likely to be incorporated (9–11). This problem was overcome by including a DNA template sequence starting with a stretch of G residues and encoding a hammerhead ribozyme upstream of the tRNA gene (12). Once transcribed, ribozyme self-cleavage generates the tRNA transcript, starting with the desired, specified nucleotide.

Recently, two efficient methods for tRNA production were proposed (13): preparation of the double-stranded DNA template by PCR or *in vitro* ligation by T4 RNA ligase of two pieces of chemically synthesized RNA. The PCR method avoids time-consuming cloning and large-scale preparation of DNA templates, but still requires the PCR step prior to the transcription reaction itself. Ligation of two pieces of RNA is straightforward and even allows incorporation of modified bases into the tRNA; however, the cost of chemically synthesized RNAs may limit its use.

Double-stranded DNA is required for recognition and efficient binding of the T7 RNA polymerase to the promoter region, although it is not imperative for extension (8,14–17). T7 RNA polymerase, in contrast to SP6 RNA polymerase (18), can accommodate single-stranded DNA template. It was shown earlier that small RNAs could be transcribed from single-stranded templates (8,14). Here we show a method for *in vitro* production of tRNA gene transcripts that eliminates the time-consuming DNA maxipreparations, restriction digestions and PCR. The transcription template is formed by annealing two oligonucleotides: the longer oligonucleotide comprises the tRNA gene and at its 3′ end a 23 nt sequence complementary to the shorter oligonucleotide. The resulting double-stranded region forms the T7 promoter (Fig. 1). The tRNA 3′-terminal CCA end is subsequently generated by run-off transcription.

MATERIALS AND METHODS

Chemicals, enzymes and oligonucleotides

Plasmid T7-911 for preparation of the His_6-tagged recombinant T7 RNA polymerase was a gift from T. Shrader (Department of Biochemistry, Albert Einstein College of Medicine, New York, NY). Purification of the *Borrelia burgdorferi* lysyl-tRNA synthetase (LysRS) and *Methanococcus jannaschii* prolyl-tRNA synthetase (ProRS) were

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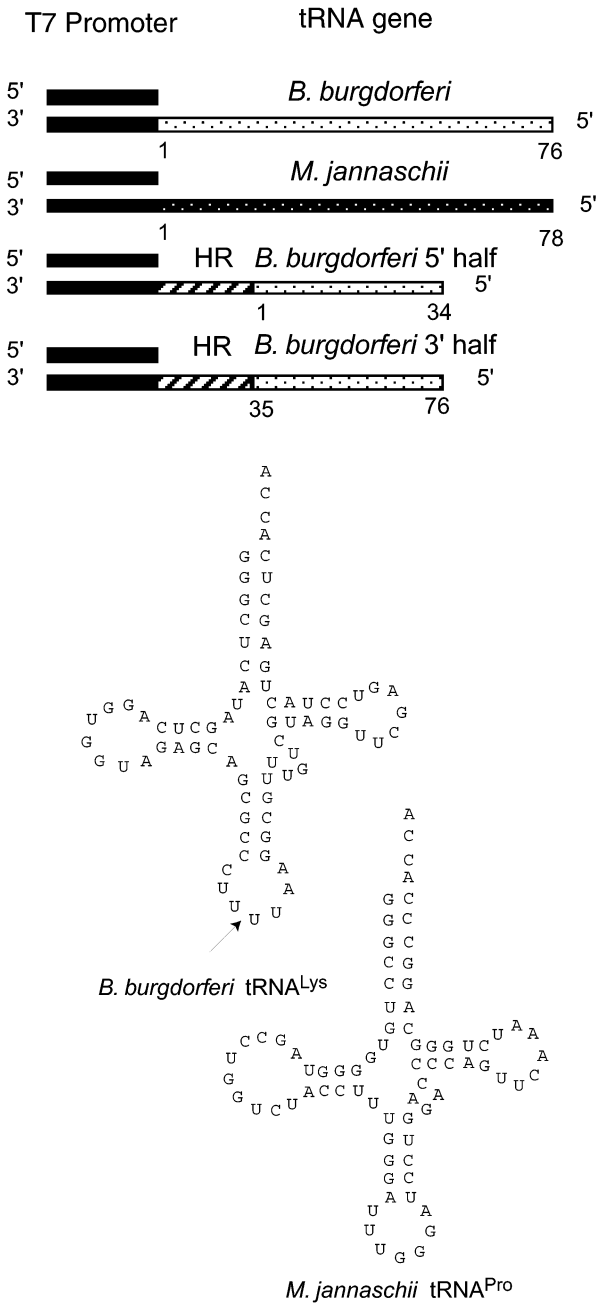

Figure 1. Diagram of DNA templates used for *in vitro* generation of tRNA gene transcripts. The shaded portion indicates the two strands of the T7 RNA polymerase promoter (23 nt). The white spotted and the black spotted portions represent sequences complementary to those shown for the *B.burgdorferi* $tRNA^{Lys}$ (76 nt for the full-length molecule, 34 and 42 nt for the 5′-half and 3′-half tRNA molecules) and *M.jannaschii* $tRNA^{Pro}$ (78 nt), respectively. The striped portion represents the transcription template encoding the hammerhead ribozyme (HR) (57 nt). The numbers represent the nucleotide positions in the tRNA sequences. The arrow indicates the ligation position of the two half tRNA molecules.

performed as described (19,20).The oligonucleotides were synthesized by the Keck Foundation Biotechnology Resource Laboratory at Yale. NTPs were purchased from Sigma.

Oligonucleotides and template preparation

The following DNA oligonucleotides were chemically synthesized (the underlined sequence indicates the double-stranded promoter region of the transcription template): T7 promoter 1 (T7.1), 5′-GGATCCTAATACGACTCACTATA-3′; T7 promoter 2 (T7.2), 5′-GGATCCTAATACGACTCACTATAGGGCT-3′; T7 promoter3 (T7.3), 5′-GGATCCTAATACGACTCACTATAGGGCTCATAG-3′; *B.burgdorferi* $tRNA^{Lys}$ (BBLys1), 5′-TGGTGAGCTCAGTAGGACTCGAACCTACGACAAACGCCTTAAAAGGGCGCTGCTCTACCACCTGAGCTATGAGCCCTATAGTGAGTCGTATTAGGATCC-3′; *M.jannaschii* $tRNA^{Pro}$ (MJPro), 5′-TGGTGGGCCTGCCCAGATTTGAACTGGGGTCTCAGGATCCCAAATCCCAAAGGATAGACCAGGCTACCCCACAGGCCCTATAGTGAGTCGTATTAGGATCC-3′.

Transcription templates for production of *B.burgdorferi* $tRNA^{Lys}$ were prepared by annealing of the tRNA encoding oligonucleotide (BBLys1) to different T7 promoter oligonucleotides (T7.1, T7.2 or T7.3). Similarly, templates for production of *M.jannaschii* $tRNA^{Pro}$ were constructed by annealing of oligonucleotide MJPro to T7.1. Annealing conditions for all the templates were as follows: a mixture of the long oligonucleotide (BBLys1 for instance, 25 µM) and of the short oligonucleotide (T7.1, 25 µM) was incubated at 95°C for 5 min and allowed to gradually cool to 25°C.

RNA ligation

The following DNA oligonucleotides were chemically synthesized (the underlined sequence indicates the double-stranded promoter region of the transcription template and the bold portion indicates the hammerhead ribozyme sequence): *B.burgdorferi* $tRNA^{Lys}$ nucleotides 1–34 (BBLys2), 5′-AAGGGCGCTGCTCTACCACCTGAGCTATGAGCCC**GACGGTACCGGGTACCGTTTCGTCCTCACGGACTCATCAGCGGCTCATAGCTCTCCC**TATAGTGAGTCGTATTAGGATCC-3′; *B.burgdorferi* $tRNA^{Lys}$ nucleotides 35–76 (BBLys3), 5′-TGGTGAGCTCAGTAGGACTCGAACCTACGACAAACGCCTTAA**GACGGTACCGGGTACCGTTTCGTCCTCACGGACTCATCAGCGGCTCATAGCTCTCCC**TATAGTGAGTCGTATTAGGATCC-3′. Oligonucleotides BBLys2 and BBLys3 were annealed to the T7 promoter oligonucleotide T7.1 and used as a transcription template as described above. Ribozyme cleavage was promoted by incubation at 60°C for 45 min. The two 'half' tRNA molecules were purified on a 12% polyacrylamide gel, extracted, ethanol precipitated and ligated using T4 RNA ligase as previously described (13). The ligation product of the expected size was then purified on a 12% polyacrylamide gel, extracted and ethanol precipitated.

Transcription

In order to determine the optimal ratio of T7 RNA polymerase to template DNA necessary for efficient transcription, variable amounts (from 0 to 2.5 µM) of the annealed oligonucleotides were tested in transcription reactions. Reactions were carried out as described before (5) in 40 mM Tris–HCl, pH 8.0, 22 mM $MgCl_2$, 1 mM spermidine, 5 mM DTT, 0.5% Triton-X100, 4 mM each NTP, 5 mM GMP and 30 nM T7 RNA polymerase for 3 h at 37°C. Aliquots (5 µl) of the transcription reaction were run on a 12% polyacrylamide gel containing 8 M urea.

The gel was stained with toluidine blue to quantify the yield of the reactions. For tRNA preparation, the transcription reaction products were phenol/chloroform extracted, ethanol precipitated, resuspended in sterile water and purified on a 12% polyacrylamide gel. The purified tRNAs were extracted from the gel as described (12).

Aminoacylation reaction

Aminoacylation reactions were performed as described before (19,20). Reaction mixtures contained 100 mM HEPES–NaOH pH 7.2, 10 mM $MgCl_2$, 50 mM KCl, 5 mM DTT, 10 mM ATP, 30 µM [^{14}C]lysine (300 c.p.m./pmol; NEN), 5 µM $tRNA^{Lys}$ transcript and 500 nM *B.burgdorferi* LysRS for 30 min at 37°C. For $tRNA^{Pro}$ transcript aminoacylation, reactions were conducted in 50 mM Tris–HCl pH 7.5, 50 mM KCl, 15 mM $MgCl_2$, 5 mM DTT, 10 mM ATP, 19 µM [^{14}C]proline (500 c.p.m./pmol; Amersham), 5 µM $tRNA^{Pro}$ transcript and 300 nM *M.jannaschii* ProRS at 60°C.

Preparation of transcripts from double-stranded plasmid DNA templates

Transcripts of *B.burgdorferi* $tRNA^{Lys}$ and *M.jannaschii* $tRNA^{Pro}$ were prepared as previously described (19,20). Plasmid DNA was obtained by large-scale preparation using the Qiagen® Plasmid Maxi Kit and digested overnight with *Bst*NI restriction enzyme (NEB). After phenol/chloroform extraction, the digested DNA was ethanol precipitated and used for *in vitro* transcription at a concentration of 0.1 mg/ml.

RESULTS AND DISCUSSION

Structural data (21,22) and biochemical evidence show that a double-stranded T7 promoter is a prerequisite for T7 RNA polymerase recognition and formation of the initiation complex. However, this is not the case for the elongation complex, as the role of the non-coding strand is limited to facilitating RNA displacement after transcription (23). T7 RNA polymerase has been shown to transcribe single-stranded DNA templates of up to 40 nt linked to the double-stranded promoter region (8,14). Therefore, we investigated the transcription properties of similar partially double-stranded DNA templates with longer single-stranded template regions sufficient to encode a tRNA gene.

Minimum double-stranded region

Given that the transcription yield depends on the stabilization that the non-coding strand contributes to the transcription complex, we investigated how an extended double-stranded region beyond the promoter would affect the reaction yield. This was tested by comparing two constructs, in which the double-stranded region extended 5 bp (BBLys1/T7.2) or 10 bp (BBLys1/T7.3) beyond the promoter (23 bp in length), with that in which only the promoter region is double stranded (BBLys1/T7.1). In line with earlier data on short RNAs (8), adding only up to 10 bases to the double-stranded DNA portion had little effect on the transcription yield of full-length tRNAs (Fig. 2). As expected, the oligonucleotide (BBLys1) without a double-stranded promoter was not a template for transcription (Fig. 2).

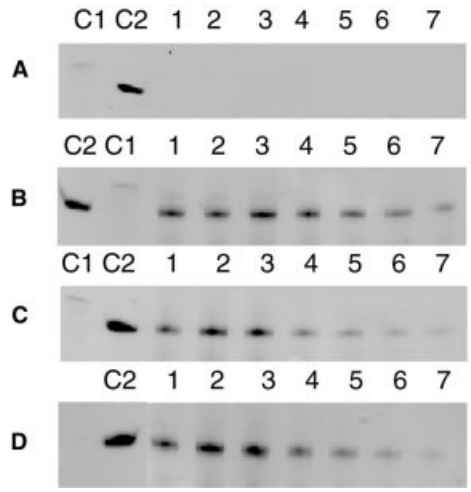

Figure 2. *In vitro* transcription of *B.burgdorferi* $tRNA^{Lys}$ with double-stranded regions of variable length. Aliquots (5 µl) of the transcription reactions were loaded on 12% polyacrylamide–8 M urea gels. Increasing amounts of template (0–2.5 µM) were used in lanes 1–7. C1, DNA transcription template (10 µl BBLys1); C2, *B.burgdorferi* $tRNA^{Lys}$ transcript (1.1 µg) obtained by transcription of digested plasmid DNA. (**A**) No double-stranded portion (BBLys1). (**B**) Only the T7 promoter is double-stranded (BBLys1/T7.1). (**C**) The double-stranded portion extends to +5 on the tRNA gene (BBLys1/T7.2). (**D**) The double-stranded portion extends to +10 on the tRNA gene (BBLys1/T7.3).

Maximum length of the single-stranded region

We showed that transcription of a 76 nt single-stranded DNA template allowed production of *B.burgdorferi* $tRNA^{Lys}$. Therefore, we investigated if a longer single-stranded DNA template could also be a suitable template for *in vitro* transcription. Because the addition of 57 nt (the hammerhead ribozyme sequence) is required in the cases of the inappropriate 5′-terminus of the mature tRNA, we tested transcription with a single-stranded DNA portion of up to 133 nt (the ribozyme sequence and the *B.burgdorferi* $tRNA^{Lys}$ gene). This did not work satisfactorily, as the yield of the expected product was low and many shorter RNA fragments were formed. Thus, we considered RNA ligation of tRNA halves (13), each generated by transcription from a single-stranded DNA of about 90 nt (the ribozyme construct and half a tRNA gene linked to the double-stranded T7 promoter; see Fig. 1). After self-cleavage of the ribozymes, the two desired RNA fragments were gel purified (Fig. 3). Ligation of the two tRNA halves with T4 RNA ligase generated a full-length tRNA-sized product (Fig. 3) that was subsequently purified and aminoacylated.

Transcription yield and biological activity of transcribed RNA

Using two different tRNA genes in multiple reactions we compared (Table 1) the amount of tRNA obtained with our method based on single-stranded DNA transcription to that derived from transcription of double-stranded DNA (5,13). The transcription yield in our method was 40–50% of that obtained with the double-stranded template procedure. Similar yield differences were reached in transcription of other tRNA genes (data not shown). This is not unexpected, as double-stranded DNA is known to be a better template for

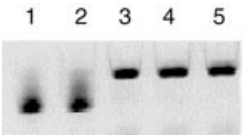

Figure 3. Production of *B.burgdorferi* $tRNA^{Lys}$ by *in vitro* ligation of the two RNA halves. Lane 1, 5′-half of tRNA (nucleotides 1–34); lane 2, 3′-half of tRNA (nucleotides 35–76); lane 3, ligation product (T4 RNA ligase) of the two half molecules of lanes 1 and 2; lanes 4 and 5, *in vitro* transcript of *B.burgdorferi* $tRNA^{Lys}$ obtained by transcription from a single-stranded or from a double-stranded DNA template, respectively.

Table 1. Comparison of transcript tRNA production methods

tRNA	Transcription yield (μg)[a]	Aminoacylation (pmol/A_{260})
B.burgdorferi $tRNA^{Lys}$		
dsDNA template	420	400
ssDNA template	200	400
M.jannaschii $tRNA^{Pro}$		
dsDNA template	350	300
ssDNA template	140	300

[a]Per 0.5 ml reaction.

transcription of short RNAs (15). Nevertheless, our procedure afforded an appreciable amount of tRNA transcript. Judged by their ability to be aminoacylated by the cognate aminoacyl-tRNA synthetase, the tRNA transcription products generated by the two different methods had equal biological activity (Table 1).

CONCLUSIONS

Single-stranded DNA templates longer than 100 nt are not suitable for *in vitro* transcription. This limitation can be overcome either by preparation of a double-stranded DNA template (by cloning or by PCR) or ligation of two tRNA half molecules obtained by transcription from a smaller single-stranded DNA template. Despite the fact that the standard *in vitro* transcription method using double-stranded DNA template yielded higher amounts of tRNA transcript, we find simplicity, quality of the transcript and cost-effectiveness to be the major advantages of the method described here. This single-stranded DNA template method should facilitate the production of the large number of tRNA mutant species that are routinely used for probing protein–RNA interactions.

ACKNOWLEDGEMENTS

We thank M. Ibba, S. Bunjun-Srihari and D. Tumbula-Hansen for constructive discussions. This work was supported by a grant from the National Institute for General Medical Sciences.

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