Volume 212, number 2, 271–275 FEB 04461 February 1987

Simplified in vitro synthesis of mutated RNA molecules

An oligonucleotide promoter determines the initiation site of T_7 RNA polymerase on ss M13 phage DNA

Guido Krupp and Dieter Söll

Department of Molecular Biophysics and Biochemistry, Yale University, PO Box 6666, New Haven, CT 06511, USA

Received 29 September 1986; revised version received 29 December 1986

We describe a simplified method for the in vitro synthesis of mutated RNA molecules. The method makes use of an oligodeoxyribonucleotide (T_7-oligo) which contains the T_7 RNA polymerase promoter sequence. In combination with a second oligonucleotide, a series of transcripts initiating and terminating at any chosen position on a cloned ss DNA (e.g. M13 phage DNA) can be generated. The phage DNA represents the non-coding DNA strand for the desired transcript; the T_7-oligo determines the transcription start site, whereas the second oligonucleotide permits the choice of the transcription termination site. The synthesis of the required template DNA is achieved by hybridizing the two oligonucleotides to the phage DNA and subsequently synthesizing the coding DNA strand by a fill-in reaction with Klenow enzyme. The reaction product is used directly as a template for T_7 RNA polymerase; cloning of mutants is not required.

T_7 RNA polymerase; Transcription; Oligonucleotide promoter

1. INTRODUCTION

The very specific RNA polymerases from the bacteriophages T_7 and SP6 [1,2] are available commercially in highly purified form. This allows the facile in vitro synthesis of RNA from cloned expression plasmids [2]. The ability to generate RNA molecules has greatly facilitated studies of RNA processing [3–7]. RNA synthesis from engineered DNA plasmids is a very powerful but labour-intensive approach, especially if a series of truncated RNAs is to be analyzed for lost or retained function [6]. Such expression plasmids are derived from vectors which contain a polylinker downstream of an RNA polymerase promoter. Frequently subcloning of the gene of interest into these vectors requires the cumbersome engineering of new restriction sites which allow the choice of start and end of the transcript. However, these manipulations may result in unwanted sequence changes. In addition, the transcripts contain the 5′-terminal stretch of the vector sequence located between the promoter and polylinker.

Here we describe the use of an oligonucleotide promoter (T_7-oligo) which circumvents these problems. In combination with a second oligonucleotide (terminator oligo) this method allows the precise initiation and termination of transcription at any point in the cloned DNA and avoids the tedious construction, characterization and subcloning of mutants.

Correspondence (present) address: Institute for General Microbiology, Universität Kiel, Olshausenstr. 40, D-2300 Kiel, FRG

Abbreviations: Klenow enzyme, Klenow fragment of DNA polymerase I; ddA, 2′,3′-dideoxyadenosine; ss, single-stranded; ds, double-stranded

Published by Elsevier Science Publishers B.V. (Biomedical Division)

2. EXPERIMENTAL

2.1. *3'-addition of ddA to the promoter oligonucleotide*

1 nmol of the promoter oligonucleotide (~20 μg) was incubated for 30 min at 25°C in 50 μl containing 7 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 50 mM NaCl, 0.1 mM ddATP and 20 U Klenow enzyme. The ddA-extended oligonucleotide (T_7-oligo) was purified on a denaturing 20% polyacrylamide gel.

2.2. *Synthesis of template DNA*

In order to permit analysis of the synthesized DNA, ^{32}P-dATP was included in this protocol, whereas RNA transcripts were obtained from unlabeled DNA. The M13 phage DNA used in this report contained a 3.4 kb *Eco*RI fragment of the K RNA gene cloned into M13mp9 [4]. 1 μg M13mp9K phage DNA (~0.3 pmol), 100 ng 5'-phosphorylated T_7-oligo (~4.5 pmol) and 3 ng of the gene specific terminator oligo GK29 (~0.3 pmol; the 'universal' or any appropriate primer can also be used) were annealed in 10 μl containing 14 mM Tris-HCl (pH 7.5), 14 mM $MgCl_2$, 100 mM NaCl, followed by incubation for 60 min at 25°C with 50 μM of each deoxynucleoside triphosphate, 0.5 μCi [α-^{32}P]dATP, 0.5 mM ATP, 0.5 mM DTT and 1 U Klenow enzyme (BRL) and 1 U DNA ligase (Promega) in a final reaction volume of 20 μl. Then the sample was heated for 5 min at 65°C.

Analysis of the reaction products by electrophoresis (8% denaturing polyacrylamide gel) and autoradiography showed that about 20% of the theoretical yield of template DNA with the expected size was obtained. Most of the product was material with a high molecular mass (not shown).

2.3. *Transcription reactions*

The synthesized template DNA was used directly or after restriction endonuclease cleavage. Non-radioactive template DNA was used. Removal of excess T_7-oligo was not necessary as no improved transcription efficiency was observed after gel electrophoretic purification (not shown). The material obtained from 0.2 μg M13 phage DNA was incubated in 10 μl of 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 500 μM NTPs (ATP, CTP, UTP), 20 μM [α-^{32}P]GTP (50 Ci/mmol) with 40 U T_7 RNA polymerase (BRL) for 60 min at 37°C. After phenol extraction, excess label was removed by ethanol precipitation from 2 M ammonium acetate (pH 7) and the product was analyzed on a denaturing 8% polyacrylamide gel.

5'-end labeled RNA for the identification of the initiating nucleoside was obtained by transcription in the presence of 100 μM [γ-^{32}P]ATP (200 Ci/mmol).

3. RESULTS

3.1. *Synthesis of the template DNA for T_7 RNA polymerase*

Transcription by T_7 RNA polymerase is well understood in terms of its promoter sequence and RNA initiation site [1]. Here we describe a method which utilizes these two features to direct transcription initiation at practically any predetermined sequence within a cloned DNA. The method has two steps: (i) the construction of the template DNA consisting of the desired sequence linked to an oligonucleotide containing the T_7 RNA polymerase promoter sequence (T_7-oligo); (ii) the transcription of the template DNA.

After choosing the DNA sequence to be transcribed (e.g., from a cloned DNA fragment in M13 phage) the T_7-oligo needs to be synthesized. This oligonucleotide (fig.1) combines the constant promoter sequence (as ds DNA in a hairpin) with a variable ss domain which is complementary to the ss phage DNA at the point where transcription should initiate. In principle, the ss domain could be at either end of the T_7-oligo. However, good transcription results were obtained only if the ss domain was at the 5'-end of the T_7-oligo, thus encoding a short segment of the 5'-terminal sequence of the desired transcript (fig.1).

The full size template DNA is synthesized in vitro, using the T_7-oligo in combination with a second oligonucleotide (terminator oligo). The complete sequence of the desired transcript is present in the phage DNA segment (non-coding strand) whose ends are defined by the two hybridized oligonucleotides (coding strand) as shown in fig.2. The complete coding DNA strand is obtained by primer extension of the terminator oligo with Klenow enzyme, closing the gap between the two hybridized oligonucleotides. In the

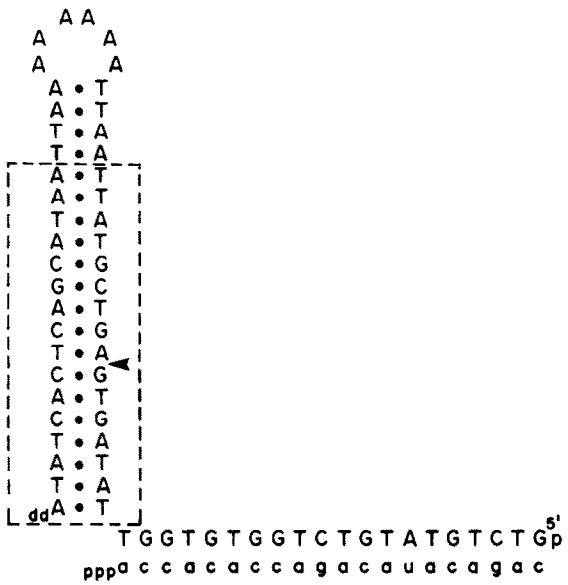

Fig.1. Sequence of the synthetic oligodeoxyribonucleotide (T_7-oligo). The T_7 promoter [1] is boxed. The encoded transcript is shown in lower case letters. The arrowhead indicates the suggested ligation point of a shortened T_7-oligo ('portable promoter') with a gene-specific oligonucleotide (see section 4).

presence of DNA ligase the synthesized DNA is joined to the 5'-phosphorylated T_7-oligo (fig.2). It is important to block the extension of the T_7-oligo by prior introduction of ddA at its 3'-end [8] (see section 2.1). Otherwise the ss domain would be converted to ds DNA and would no longer be available for hybridization to M13 DNA. The resulting template DNA contains the T_7 promoter sequence in a hairpin structure (i.e. as ds DNA), joined to the in vitro synthesized coding strand for the desired transcript (fig.2). This construct, still hybridized to the phage DNA, can be used directly as a template for transcription.

The method was tested in the experiment shown in fig.3. Transcription was expected to initiate precisely at the first nucleotide in the ss domain of the T_7-oligo (fig.1) and terminate at the end of the synthesized DNA. Thus, the 3'-terminal nucleotide of the transcript is encoded by the 5'-terminal nucleotide of the terminator oligo (GK29). Alternatively, the standard protocol for the termination of run-off transcription can be used, so that cleavage of the DNA by a convenient restriction endonuclease determines the 3'-end of the transcript (*TaqI* and *RsaI* in fig.3).

3.2. *The transcription initiation site is determined precisely by the T_7-oligo*

In order to confirm that the predicted transcrip-

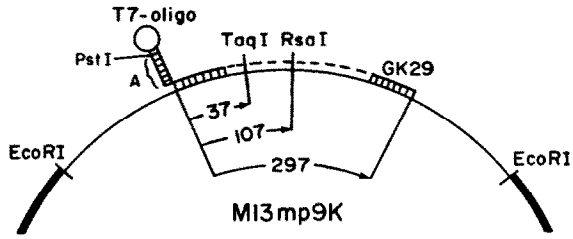

Fig.3. A specific example of RNA synthesis. For details see text. M13 DNA (thick line) containing an inserted DNA fragment (thin line) is shown. The synthesized template DNA is represented as a broken line. Restriction sites are marked and the lengths and orientations of the expected transcripts are indicated. If cloning of the template DNA is desired, an extended T_7-oligo is used, which includes the indicated *PstI* recognition sequence (not present in the T_7-oligo shown in fig.1). *PstI* will be used to generate a ss DNA fragment which is converted to ds DNA by primer-extension of oligonucleotide A (for details see section 4).

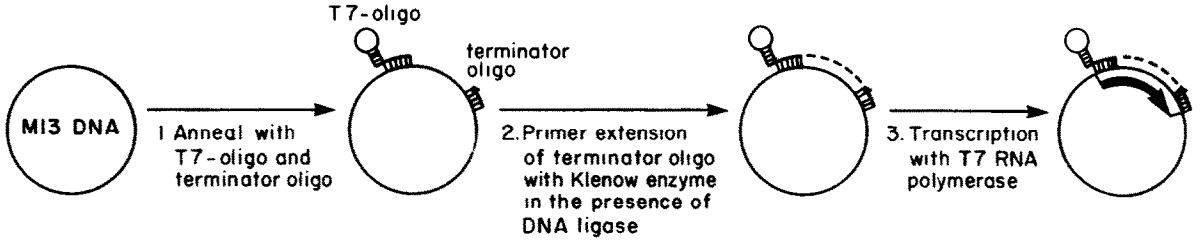

Fig.2. Scheme for the synthesis of the template DNA for T_7 RNA polymerase transcriptions. The arrowheads indicate the 3'-ends of terminator oligo and RNA transcript.

tion initiation site was actually used, the size of two short transcripts was determined. The synthesized template DNA was cleaved with *Rsa*I or *Taq*I (converted to blunt-ends with Klenow enzyme) and the subsequent transcription reactions yielded the expected RNAs of 107 or 37 nucleotide length, respectively (figs 3, 4A, 4B). In addition, the 5′-terminal nucleoside was identified. As indicated in fig.1, initiation with an adenosine residue was expected. Accordingly, internal labeling with [α-^{32}P]GTP, or labeling of only the initiating nucleotide with [γ-^{32}P]ATP resulted in transcripts of the same size (fig.4).

3.3. *Oligonucleotides allow precise initiation and termination of RNA synthesis*

As reported by Eperon [9], oligonucleotides permit transcription termination at a predetermined site. In his approach, however, the ss phage DNA already contains the T_7 RNA polymerase promoter sequence, covalently joined to the non-coding strand for the desired transcript. This phage DNA is derived from mICE [9], Bluescribe or similar vectors. A terminator oligo hybridizes to the DNA where transcription should terminate; the coding strand is synthesized by primer extension with Klenow enzyme. In these constructs primed DNA synthesis is extended beyond the promoter sequence, which assures that the promoter is present as ds DNA. This is mandatory for the recognition by RNA polymerase [1]. However, it should be noted that the coding DNA strand itself can be used in ss form. The isolated T_7-oligo codes for a short transcript (indicated in fig.1), which can be synthesized with T_7 RNA polymerase (not shown).

The T_7-oligo directed transcription initiation described above was combined with oligonucleotide-directed transcription termination. As the terminator oligo (see section 3.1), the gene specific oligonucleotide GK29 was used as the primer in template DNA synthesis (fig.3). The obtained DNA construct was not cleaved with restriction endonucleases and transcription terminated at the expected site (fig.3): a 297 nucleotide long RNA was synthesized by T_7 RNA polymerase (fig.4C); ~90000 cpm from 0.2 μg ss M13 DNA (~10 kb).

This amount of labeled RNA corresponds to about 10% of the yield obtained under similar conditions with a standard DNA template, i.e. 0.2 μg

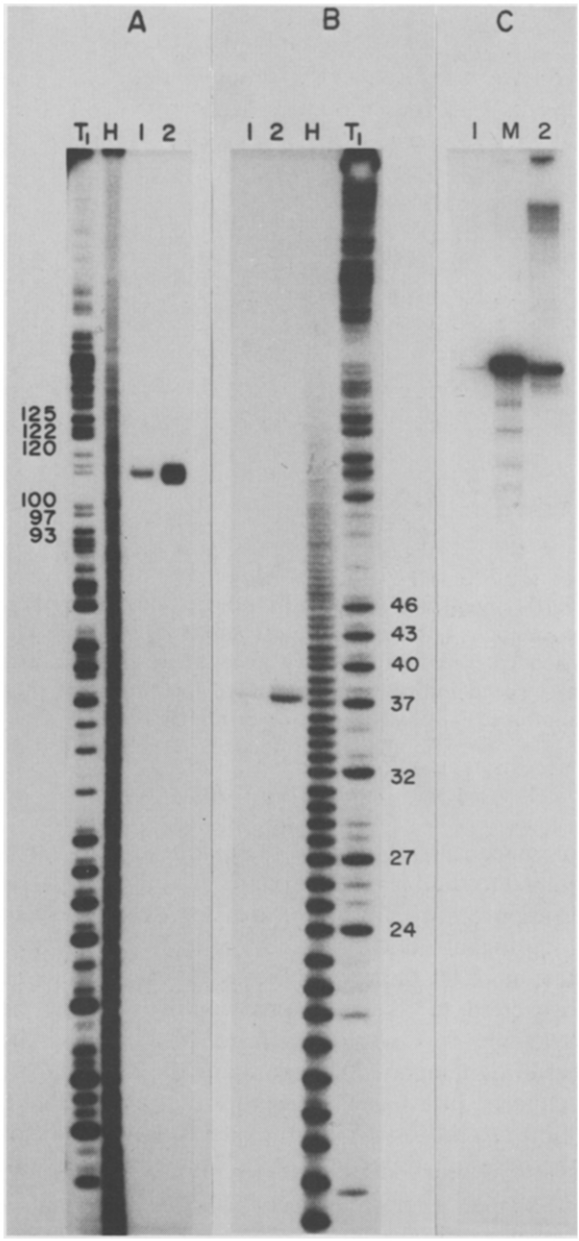

Fig.4. Analysis of the transcripts. Panels A and B: run-off transcripts from *Rsa*I and *Taq*I cleaved template DNA, respectively. Panel C: product of GK29-directed transcription termination. Lanes: 1,2, transcripts with [γ-^{32}P]ATP or [α-^{32}P]GTP, respectively; T_1, H, size markers obtained from a 5′-^{32}P-labeled run-off transcript (linearized pGem3 derivative as template) after RNase T_1 or acid cleavage [4]; M, 301 nucleotide run-off transcript as size marker.

linearized expression plasmid (~3.5 kb). However, only about 20% of the M13 phage DNA used was converted to ds template DNA (section 2.2). This means that the amount of transcript synthesized may reach up to 50% of the value obtained, if compared to the same molar amount of standard expression plasmid.

In addition to the expected product, undefined large RNAs were also synthesized (fig.4C, lane 2). This is a common problem if ds DNA with a 3′-protruding end is used as a template for RNA polymerases and was also observed in oligonucleotide-directed transcription termination ([9] and Eperon, I.C., personal communication).

4. DISCUSSION

Our method for the simplified in vitro synthesis of mutated RNA molecules is based on the use of an oligonucleotide hairpin structure, which serves as a promoter for T_7 RNA polymerase. In another example for the use of self-complementary oligonucleotides, it was possible to 'place' a restriction enzyme recognition site at any position in a particular DNA sequence [10].

Our scheme has several advantages over the more conventional cloning and subcloning of engineered expression plasmids. (i) RNAs which begin and end at any chosen point of a cloned DNA fragment are easily available. (ii) The chemical synthesis of oligodeoxyribonucleotides which are to be incorporated into the coding DNA strand allows the easy introduction of point mutations near both termini of the RNA transcript. (iii) The requirements for oligonucleotide synthesis are not excessive, since the long hairpin structure containing the T_7 RNA polymerase promoter is synthesized only once ('portable promoter') and can be ligated to any gene-specific oligonucleotide (see arrow in fig.1).

However, if a particular construct is needed frequently, cloning into a convenient vector may be preferable. For this purpose the extended T_7-oligo is used. It contains a *Pst*I recognition site in the ds hairpin (indicated in fig.3). The ^{32}P-labeled template DNA is synthesized as described (see sections 2.2 and 3.1) and cut with *Pst*I. Then the labeled ss DNA fragment is isolated after denaturing gel electrophoresis. After annealing with oligonucleotide A (fig.3; without 3′-terminal ddA), primer extension with reverse transcriptase is possible. Reverse transcriptase is required because it lacks a 3′-5′-exonuclease activity [11]. Thus the 3′-protruding end generated by *Pst*I is retained in the newly generated ds DNA fragment. This allows convenient cloning into an appropriate vector for large scale preparations of plasmid containing the template DNA insert.

ACKNOWLEDGEMENTS

We are indebted to Gary O'Neill, Astrid Schön and Ian Willis for their critical reading of the manuscript. This work was supported by a grant from the National Institutes of Health.

REFERENCES

[1] Osterman, H.L. and Coleman, J.E. (1981) Biochemistry 20, 4884–4892.
[2] Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K. and Green, M.R. (1984) Nucleic Acids Res. 12, 7035–7056.
[3] Frendewey, D. and Keller, W. (1985) Cell 42, 355–367.
[4] Krupp, G., Cherayil, B., Frendewey, D., Nishikawa, S. and Söll, D. (1986) EMBO J. 5, 1697–1703.
[5] Reich, C., Gardiner, K.J., Olson, G.J., Pace, B., Marsh, T.L. and Pace, N.R. (1986) J. Biol. Chem. 261, 7888–7893.
[6] Guerrier-Takada, C. and Altman, S. (1986) Cell 45, 177–183.
[7] Garriga, G., Lambowitz, A.M., Inoue, T. and Cech, T. (1986) Nature 322, 86–89.
[8] Deutscher, M. and Kornberg, A. (1969) J. Biol. Chem. 244, 3019–3028.
[9] Eperon, I.C. (1986) Nucleic Acids Res. 14, 2830.
[10] Podhajska, A.J. and Szybalski, W. (1985) Gene 40, 175–182.
[11] Battula, N. and Loeb, L.A. (1976) J. Biol. Chem. 251, 982–986.