Video Article

Substrate Generation for Endonucleases of CRISPR/Cas Systems

Judith Zoephel[1], Srivatsa Dwarakanath[1], Hagen Richter[1], André Plagens[1], Lennart Randau[1]

[1]Prokaryotic Small RNA Biology, Max-Planck-Institute for Terrestrial Microbiology

Correspondence to: Lennart Randau at lennart.randau@mpi-marburg.mpg.de

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Abstract

The interaction of viruses and their prokaryotic hosts shaped the evolution of bacterial and archaeal life. Prokaryotes developed several strategies to evade viral attacks that include restriction modification, abortive infection and CRISPR/Cas systems. These adaptive immune systems found in many Bacteria and most Archaea consist of clustered regularly interspaced short palindromic repeat (CRISPR) sequences and a number of CRISPR associated (Cas) genes **(Fig. 1)** [1-3]. Different sets of Cas proteins and repeats define at least three major divergent types of CRISPR/Cas systems [4]. The universal proteins Cas1 and Cas2 are proposed to be involved in the uptake of viral DNA that will generate a new spacer element between two repeats at the 5' terminus of an extending CRISPR cluster [5]. The entire cluster is transcribed into a precursor-crRNA containing all spacer and repeat sequences and is subsequently processed by an enzyme of the diverse Cas6 family into smaller crRNAs [6-8]. These crRNAs consist of the spacer sequence flanked by a 5' terminal (8 nucleotides) and a 3' terminal tag derived from the repeat sequence [9]. A repeated infection of the virus can now be blocked as the new crRNA will be directed by a Cas protein complex (Cascade) to the viral DNA and identify it as such via base complementarity[10]. Finally, for CRISPR/Cas type 1 systems, the nuclease Cas3 will destroy the detected invader DNA [11,12].

These processes define CRISPR/Cas as an adaptive immune system of prokaryotes and opened a fascinating research field for the study of the involved Cas proteins. The function of many Cas proteins is still elusive and the causes for the apparent diversity of the CRISPR/Cas systems remain to be illuminated. Potential activities of most Cas proteins were predicted via detailed computational analyses. A major fraction of Cas proteins are either shown or proposed to function as endonucleases [4].

Here, we present methods to generate crRNAs and precursor-cRNAs for the study of Cas endoribonucleases. Different endonuclease assays require either short repeat sequences that can directly be synthesized as RNA oligonucleotides or longer crRNA and pre-crRNA sequences that are generated via *in vitro* T7 RNA polymerase run-off transcription. This methodology allows the incorporation of radioactive nucleotides for the generation of internally labeled endonuclease substrates and the creation of synthetic or mutant crRNAs. Cas6 endonuclease activity is utilized to mature pre-crRNAs into crRNAs with 5'-hydroxyl and a 2',3'-cyclic phosphate termini.

Video Link

The video component of this article can be found at http://www.jove.com/video/4277/

Protocol

1. Generation of Long Pre-crRNA Substrates via PCR

1. Design PCR primers targeting the spacer regions of a CRISPR cluster. Add the T7 RNA polymerase (T7RNAP) promoter sequence (5' -taatacgactcactata-3') to the forward primer and restriction sites for cloning the PCR product into a vector to both primers (*e.g.* BamHI and Hind III for pUC19, **Fig. 2A**).
   Note: The T7RNAP requires a guanidine residue for proper initiation of transcription.
2. Amplify your pre-crRNA sequence of interest from genomic DNA by PCR.
3. Separate the PCR products by agarose gel electrophoresis and gel extract the desired band. Digest the PCR product with the restriction enzymes to create sticky ends (*e.g.* BamHI and HindIII, **Fig. 2A**). Purify your PCR product with a PCR purification kit to eliminate cleavage by-products.
4. Set up a ligation reaction that contains T4 DNA ligase, T4 DNA ligase buffer and a 3:1 molar ratio of the cleaved PCR product and the dephosphorylated linear pUC vector with corresponding sticky ends. Incubate the mixture at 16 °C overnight. Transform the ligation mixture into competent *Escherichia coli* DH5α cells by standard protocols and use blue white screening to identify successful ligation.
5. Isolate plasmids from white colonies using a plasmid preparation kit. Identify positive clones by plasmid sequencing. Alternatively, colony PCR might be utilized for screening.

2. Generation of Intermediate Pre-crRNA Substrates via Annealing of DNA Oligonucleotides

1. Design forward and reverse oligonucleotides with the desired CRISPR repeat/spacer sequence. The oligonucleotides contain the sequence of a T7 RNAP promoter as well as terminal restriction sites (*e.g.* BamHI and Hind III for pUC19). Terminate the oligonucleotides to ensure that sticky ends form after annealing (see **Fig. 2B**).
2. 5'-phosphorylate 1 nmol of each oligonucleotide in a separate 20µl reaction containing 5 µl of T4 polynucleotide kinase (PNK), 2 µl of T4 PNK 10x buffer, 2 µl ATP (10 mM). Incubate each sample for 1 hour at 37 °C.
3. Hybridize the two phosphorylated oligonucleotides. Combine 1µl of the phosphorylated forward oligo mixture (from 2.2.), 1 µl of the phosphorylated reverse oligo mixture (from 2.2.), 1 µl of T4 DNA ligase 10x buffer in a 10 µl reaction. Incubate the samples for 5 min at 95 °C on a heating block or in boiling water, turn off the heat source and let the mixture cool down to room temperature (~2-3 hours).
   Note: In this critical step, the slow cooling process favors the annealing of the two oligonucleotides compared to the formation of structures within each single oligonucleotide.
4. Ligate 4 µl of the hybridization mix, 1µl of digested and dephosphorylated pUC vector (0.1 µg/µl) with T4 DNA ligase, T4 DNA ligase 10x buffer and 10 mM ATP in a 20 µl ligation mixture. Incubate the sample at 16 °C overnight.
5. Transform the ligated plasmids into competent *Escherichia coli* DH5α cells by standard protocols and utilize blue white screening. Isolate plasmids and identify positive clones by digestion (to screen for inserts of the desired size) and subsequent plasmid sequencing.

3. Generation of Short Cas RNA Substrates via Custom RNA Oligonucleotide Synthesis

Design short Cas RNA substrates (*e.g.* single repeat sequences, **Fig. 2C**) and utilize custom RNA oligonucleotide synthesis facilities.

Note: The inclusion of a deoxyribonucleotide at a specified position of an RNA oligonucleotide (**Fig. 2C**) can be used to pinpoint the site of RNA cleavage.

4. *In vitro* T7 RNA Polymerase Transcription

1. Isolate plasmids with your designed construct (from 1.9. or 2.7.) using a maxiprep plasmid purification kit.
2. Linearize the plasmid with the restriction enzyme that cleaves downstream of the cloned fragment (*e.g.* HindIII). Ensure complete digestion.
   Note: If a divergent defined 3' terminus of the RNA transcript is desired, the designed construct should contain an additional specific restriction site for "run-off" transcription upstream of the HindIII sequence.
3. Purify the linearized plasmid by phenol:chloroform (1:1) extraction and ethanol precipitation. Recover the nucleic acids by resuspending the pellet in DEPC treated sterile water.
4. Set up an *in vitro* T7 RNAP run off transcription mixture that contains 40 mM Hepes/KOH (pH 8), 22 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM spermidine, 4 mM of each nucleoside triphosphate (ATP, CTP, GTP, UTP), 40-100 µg/ml of digested plasmid and 0.1 mg/ml T7 RNAP in DEPC treated water. Incubate for 3 hours at 37 °C.
5. Analyze the obtained RNA transcripts on a denaturing 8 M urea 12% polyacrylamide gel (**Fig. 3A**). The RNA transcripts can be purified via Mono Q anion exchange chromatography[13] and recovered by ethanol precipitation of the RNA fractions and resuspension of the pellet in DEPC treated sterile water. For future use, store the RNA at -80 °C.

5. Cas6 Endonuclease Assay

1. Set up a 20 µl *in vitro* T7 RNAP run off transcription mixture (see 4.4) that contains a reduced amount of 2 mM ATP and is complemented with 2.5 µl α-[^{32}P]-ATP (10 mCi/ml, 5000 Ci/mmol). Purify reaction products via gel extraction from a denaturing 8 M urea 12% polyacrylamide gel. Visualize bands by autoradiography.
2. Produce and purify the desired recombinant Cas proteins. In this example, Cas6 from *Clostridium thermocellum* was purified via heat precipitation and Ni-NTA chromatography.
3. Set up an endonuclease assay reaction (*e.g.* for *Clostridium thermocellum* Cas6, the reaction mixture contains 20 mM Hepes (KOH pH8), 250 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 12,000 cpm RNA substrate and 1 µM enzyme and was incubated at 37 °C for 30 min).
4. Load 5 µl of the reaction mixture (+ 10 µl RNA loading buffer containing 95% formamide) on a 8 M urea 12% polyacrylamide gel. Visualize the cleavage products after electrophoresis by autoradiography.

6. Representative Results

An example of RNA substrates for the analysis of Cas endonuclease activity is shown in Figure 3A. An aliquot of 5 µl of an analytical 100 µl *in vitro* transcription reaction were loaded. Please note that the efficiency of RNA production varies between different constructs. Some factors that were observed to influence the amount of obtained RNA are (i) the initial sequence following the +1G required for transcription initiation, (ii) the possibility of RNA structure formation during transcription and (iii) the choice of the restriction site for the generation of the run-off cleavage position.

The investigation of RNA endonuclease activity requires both highly purified recombinant Cas proteins (**Fig. 3B**) and proper negative controls. Ideally, this negative control sample differs as little as possible from the investigated Cas endonuclease reaction. This can be achieved by incubation of the RNA with reaction buffer and cell-lysate without Cas expression (and following the identical purification procedure). An ideal negative control is the addition of a deoxyribonucleotide at the proposed cleavage site. In Figure 3C, the cleavage of a 5' terminal labeled repeat sequence is shown for *Clostridium thermocellum* Cas6. Under identical conditions, this repeat is not a substrate anymore when a deoxyribonucleotide is introduced at position -9. This method also provides information about the cleavage site. Finally, a long internally labeled pre-crRNA is cleaved by Cas6 and two cleavage fragments are observed.

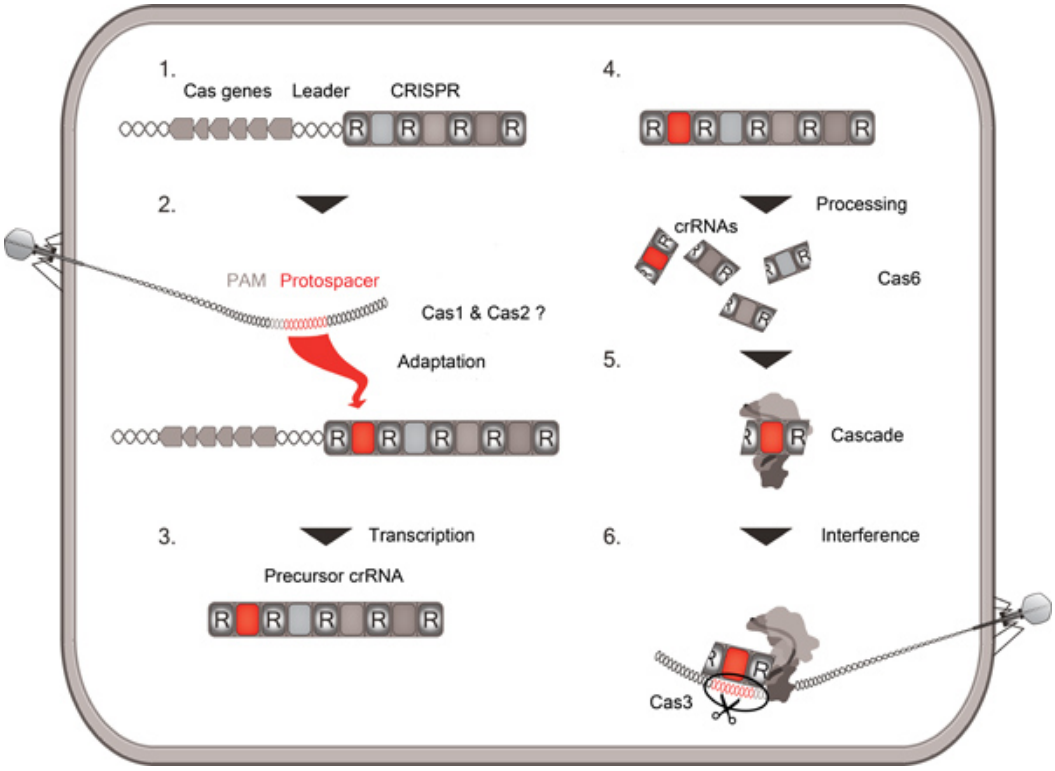

Figure 1. Schematic overview of CRISPR/Cas activity. The overview follows the insertion of a viral DNA sequence (protospacer) into the CRISPR cluster (adaptation), the transcription and processing of the CRISPR array into small crRNAs by a Cas6 endonuclease, the uptake of crRNAs into the Cascade complex and the interference of a repeated viral attack based on complementarity between crRNA and protospacer. Protospacer adjacent motifs (PAM) mark viral protospacer sequences.

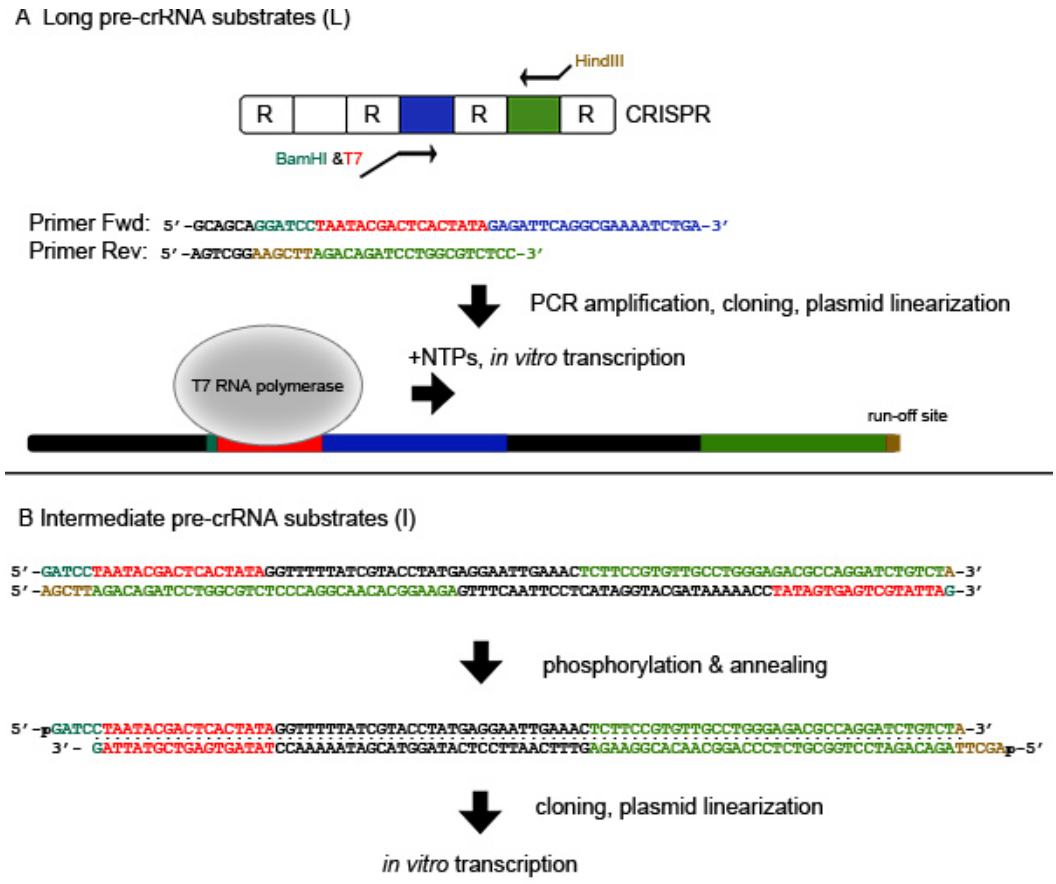

Figure 2. Generation of RNA substrates for Cas proteins. The scheme shows the workflow for generating (A) long pre-crRNA substrates, (B) intermediate pre-crRNA substrates and (C) short Cas RNA substrates for pre-crRNA production. Example sequences are presented for the CRISPR array of *Clostridium thermocellum*. Click here to view larger figure.

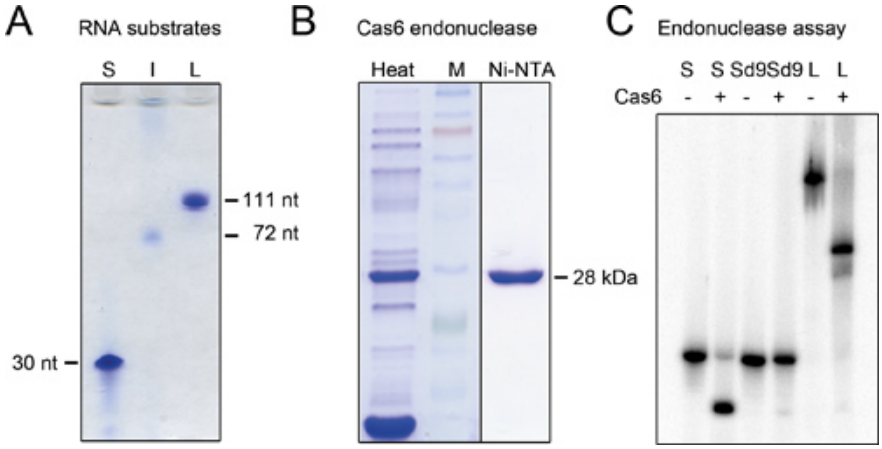

Figure 3. Cas6 endonuclease assay. A.) Toluidine blue stained polyacrylamide gel of a custom-designed RNA oligonucleotide (250 pmol) and two *in vitro* RNA transcripts (5 µl of a typical 100 µl reaction). B.) SDS-PAGE gel of a Cas6 preparation (80 pmol) from *Clostridium thermocellum* after heat precipitation at 50°C for 1 hour and Ni-NTA chromatography. C.) Detection of endonucleolytic Cas6 activity for 5' terminal labeled repeat sequences and pre-crRNA *in vitro* transcripts. The introduction of a dNTP at position -9 abolishes Cas6 cleavage for a short Cas RNA substrate (S, **Fig. 2C**). A 5' terminal 8 nt tag is also generated for crRNA maturation from long pre-crRNA substrates (L, **Fig. 2A**). The bands were separated on a denaturing 8 M urea 12% polyacrylamide gel and visualized by autoradiography.

Discussion

The presented methods enable the generation of Cas endonuclease substrates of different size ranges and with varying freedom in sequence design. The most straight-forward approach for the generation of synthetic RNA oligonucleotide substrates is limited to short RNA designs due to increasing costs and technical limitations in creating longer RNA oligomers. While successful RNA synthesis has been reported for unmodified RNA oligomers of over 100 nucleotides length, the practical and economical maximum for custom RNA synthesis lies below 40 nucleotides. However, any given sequence can be synthesized and the targeted introduction of modified nucleotides (e.g. deoxyribonucleotides) can be utilized to analyze cleavage sites in detail. Longer pre-cRNAs should be generated via *in vitro* transcription.

The annealing of oligonucleotides that contain a T7 RNA polymerase promoter allows for the production of intermediate length pre-crRNAs. The maximum length of routinely and economically synthesized DNA oligonucleotides is just above 150 nucleotides and represents the maximum of synthetic pre-crRNAs via this method. The assembly of several annealed DNA oligonucleotide pairs that form sticky ends with each other can extend this maximal length but necessitates increasing challenges in the cloning of the construct. The main advantage of this method is the capability to generate pre-crRNA constructs (and therefore Cas endonuclease substrates) with any desired sequence. This allows the testing of synthetic crRNA designs.

Finally, larger pre-crRNAs can be obtained from PCR amplificates of entire genomic CRISPR elements or fractions thereof. Alterations to the *in vitro* transcription template plasmid can be introduced via site-directed mutagenesis for the generation of pre-crRNA variants. These constructs can be used to analyze the global endonucleolytic cleavage pattern within an entire pre-crRNA.

The pioneer work for the production of unmodified RNA via T7 RNA polymerase *in vitro* transcription was based on transfer RNAs [14] and brome mosaic virus RNA [15]. The consensus T7 RNA polymerase promoter consists of a recognition domain (-17 through -5) and an initiation domain (-4 through +6) with transcription initiation at an essential guanosine +1 [16]. Positions downstream of +1 can be varied which allows for the transcription of almost any desired RNA sequence. The presented methods for generating *in vitro* run-off transcription templates allow for *in vitro* synthesis of complete pre-crRNAs that match genomic CRISPR regions or synthetic pre-crRNA variants. The transcripts are generated with a 5'-terminal triphosphate present unless the transcription reaction is primed with GMP to obtain 5' monophosphate termini [14]. Such termini are required if the transcripts are to be labeled with T4 polynucleotide kinase and γ-[^{32}P]-ATP. Cleavage activity of Cas6 and Cas6-like enzymes generates crRNA that contain 5'-hydroxyl and a 2',3'-cyclic phosphate termini. These RNAs can then be analyzed for recognition by the Cascade complex.

Disclosures

No conflicts of interest declared.

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References

1. Barrangou, R., *et al.* CRISPR provides acquired resistance against viruses in prokaryotes. *Science*. **315**, 1709-1712 (2007).
2. Makarova, K.S., Grishin, N.V., Shabalina, S.A., Wolf, Y.I., & Koonin, E.V. A putative RNA-interference-based immune system in prokaryotes: computational analysis of the predicted enzymatic machinery, functional analogies with eukaryotic RNAi, and hypothetical mechanisms of action. *Biol. Direct.* **1**, 7 (2006).
3. Lillestøl, R.K., Redder, P., Garrett, R.A., & Brugger, K. A putative viral defence mechanism in archaeal cells. *Archaea.* **2**, 59-72 (2006).
4. Makarova, K.S., *et al.* Evolution and classification of the CRISPR-Cas systems. *Nat. Rev. Microbiol.* **9**, 467-477 (2011).
5. Brouns, S.J., *et al.* Small CRISPR RNAs guide antiviral defense in prokaryotes. *Science* **321**, 960-964 (2008).
6. Carte, J., Pfister, N.T., Compton, M.M., Terns, R.M., & Terns, M.P. Binding and cleavage of CRISPR RNA by Cas6. *RNA* **16**, 2181-2188 (2010).
7. Haurwitz, R.E., Jinek, M., Wiedenheft, B., Zhou, K., & Doudna, J.A. Sequence- and structure-specific RNA processing by a CRISPR endonuclease. *Science* **329**, 1355-1358 (2010).
8. Gesner, E.M., Schellenberg, M.J., Garside, E L., George, M.M., & Macmillan, A M. Recognition and maturation of effector RNAs in a CRISPR interference pathway. *Nat. Struct. Mol. Biol.* **18**, 688-692 (2011).
9. Carte, J., Wang, R., Li, H., Terns, R.M., & Terns, M.P. Cas6 is an endoribonuclease that generates guide RNAs for invader defense in prokaryotes. *Genes Dev.* **22**, 3489-3496 (2008).
10. Wiedenheft, B., *et al.* Structures of the RNA-guided surveillance complex from a bacterial immune system. *Nature* **477**, 486-489 (2011).
11. Garneau, J.E., *et al.* The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature* **468**, 67-71 (2010).
12. Sinkunas, T. *et al.* Cas3 is a single-stranded DNA nuclease and ATP-dependent helicase in the CRISPR/Cas immune system. *EMBO J.* **30**, 1335-1342 (2011).
13. Jahn, M.J., Jahn, D., Kumar, A.M., & Söll, D. Mono Q chromatography permits recycling of DNA template and purification of RNA transcripts after T7 RNA polymerase reaction. *Nucleic Acids Res.* **19**, 2786 (1991).
14. Sampson, J.R. & Uhlenbeck, O.C. Biochemical and physical characterization of an unmodified yeast phenylalanine transfer RNA transcribed *in vitro*. *Proc. Natl. Acad. Sci. USA* **85**, 1033-1037 (1988).
15. Dreher, T. W., Bujarski, J.J. & Hall, T.C. Mutant viral RNAs synthesized in vitro show altered aminoacylation and replicase template activities. *Nature* **311**, 171-175 (1984).

16. McGinness, K. E. & Joyce, G. F. Substitution of ribonucleotides in the T7 RNA polymerase promoter element. *J. Biol. Chem.* **277**, 2987-2991 (2002).