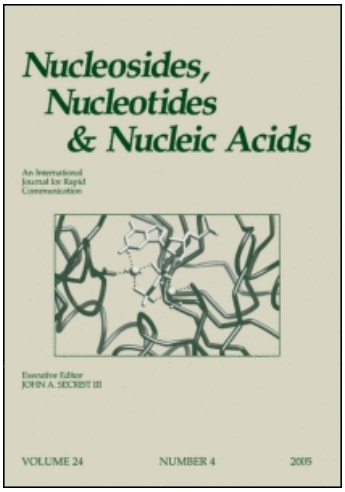

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Ribonuclease T1 Cleaves RNA After Guanosines Within Single-Stranded Gaps of Any Length

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RIBONUCLEASE T1 CLEAVES RNA AFTER GUANOSINES WITHIN SINGLE-STRANDED GAPS OF ANY LENGTH

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ABSTRACT: RNA-oligonucleotides with defined single-stranded stretches were designed to investigate the minimal requirements of a ribonuclease T1 substrate. It could be shown, that RNase T1 cleaves single-stranded RNA after a unique guanosine flanked by two double-stranded areas. However, the turnover of such a G-gap is significantly lower than that of a gap of two, three or four nucleotides.

Ribonuclease T1 (RNase T1; EC 3.1.27.3) is a single domain protein of 11,085 Da, which cleaves single-stranded RNA showing high specificity for guanosine with a ratio G : A : C/U of about 10^8 : 10^2 : 1. It is one of the most studied model enzymes with respect to the catalytic mechanism, specificity, stability and folding behavior. Several crystal structures of RNase T1 and variants in complex with the inhibitor 2'-GMP, substrate analogues, the reaction product 3'-GMP as well as of the free enzyme are available. In combination with the kinetic characterization of a variety of active and inactive variants, these structures have permitted a deeper insight into the mechanism of substrate recognition and the role of the catalytic residues of RNase T1 (for review see 1, 2). The interactions of mononucleotides and a few dinucleoside-monophosphate analogues with RNase T1 are well known (3, 4), however, detailed information on the binding of a longer RNA chain is not available.

We recently have determined the essential nucleic acid backbone conformation (FIG. 1) of a guanosine within an RNA chain of at least three nucleotides for a productive binding to RNase T1 (5). Furthermore we have shown that this conformation cannot be realized by the guanosines in a GGCA-tetraloop. Based on the results

presented in the report cited, we decided to narrow the distances of the RNase T1 cleavage point to double stranded regions down to a minimum. Korn *et al.* have demonstrated, that RNase T1 can cleave in a gap of five single-stranded nucleotides in a gapped heteroduplex (6). We therefore designed gaps of one to four nucleotides for the investigations presented in this paper.

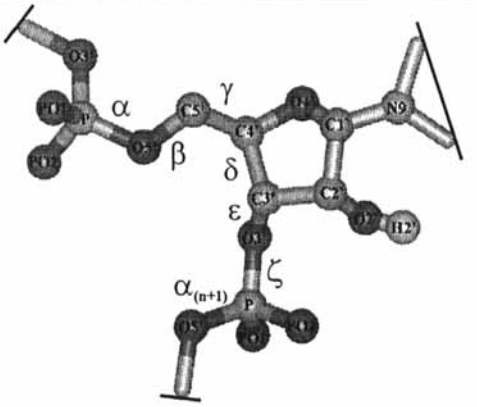
MATERIALS AND METHODS

Synthesis of the RNA-oligonucleotides. The following DNA-oligonucleotides were purchased from MWG Biotech, Ebersberg, Germany:

Primer A:	5′-TAATACGACTCACTATAGGGTCGCACCTGCGTCCC-3′
Primer B:	5′-CCAGAACACAGGTGTGTACTGG-3′
Primer B_AG:	5′-CCAGTACACAGGTGTGTCCTGG-3′
G_1:	5′-GGGTCGCACCTGCGTCCCGCCAGTACACACCTGTGTTCTGG-3′
G_2:	5′-GGGTCGCACCTGCGTCCCGGCCAGTACACACCTGTGTTCTGG-3′
G_3:	5′-GGGTCGCACCTGCGTCCCGGGCCAGTACACACCTGTGTTCTGG-3′
G_4:	5′-GGGTCGCACCTGCGTCCCGGGGCCAGTACACACCTGTGTTCTGG-3′
A_1:	5′-GGGTCGCACCTGCGTCCCACCAGTACACACCTGTGTTCTGG-3′

Enzymes, the NTPs and dNTPs were from Life Technologies, Eggenstein, Germany, Promega, Madison, USA and Boehringer Mannheim, Mannheim, Germany, respectively. The enzymatic reactions were carried out as described by the suppliers.

Firstly primers A and B and the oligonucleotides G_1, _2, _3 or _4, were used in PCRs, respectively (annealing temperatures were 60 °C), to obtain DNA-templates for the T7 RNA polymerase transcription (FIG. 2). Additionally DNA-templates were amplified using the oligonucleotide A_1 and the primers A and B or primer B_AG, respectively. The PCR products were purified on a 4 % agarose gel. 1 pmol of each PCR product was used for the transcription for at least 4 h at 37 °C. After digestion of the DNA with DNase for 1 h at 37 °C the RNA was denatured for 10 min at 95 °C and quickly cooled down to room temperature on ice. The obtained RNA samples were used directly in the various digestion assays.



Torsion angle		RNase T1	RNase A
O5'-C5'-C4'-C3'	(γ)	-62° ± 13°	-55° ± 13°
C5'-C4'-C3'-O3'	(δ)	110° ± 6°	154° ± 10°
C4'-C3'-O3'-P	(ε)	-170° ± 4°	-142° ± 5°
C3'-O3'-P-O5'$_{(n+1)}$	(ζ)	42° ± 7°	45° ± 10°

FIG. 1: Nucleic acid backbone conformation. The torsion angles defining the nucleic acid backbone conformation (10) are shown. The table shows the torsin angle ranges obtained for an productive binding of a guanosine residue in a longer RNA-chain to RNase T1 using geometry optimizations of an complex of RNase T1 and a trinucleoside-tetraphosphate (5).

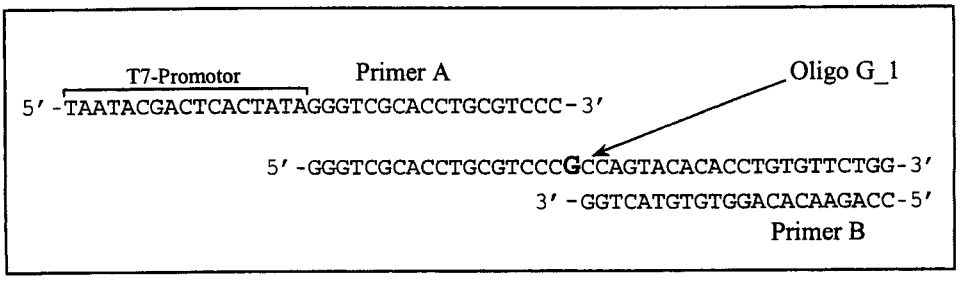


FIG. 2: Scheme of the performed PCRs . Shown are the oligonucleotide G_1, as an example, and the complementary primers A and B. With primer A the T7-promotor for the subsequently T7 RNA polymerase transcription was introduced into the PCR product, too.

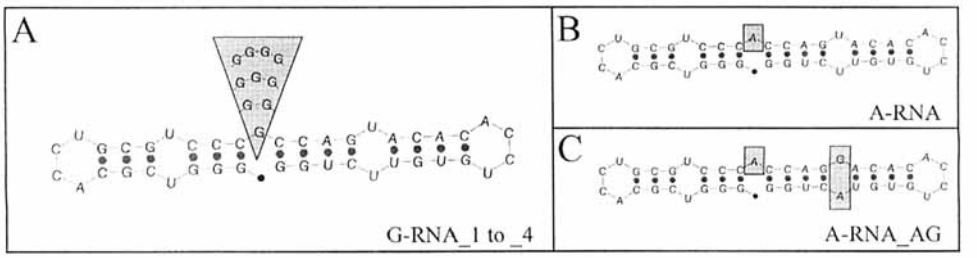


FIG. 3: Secondary structures of the designed RNA-oligonucleotides. Panel **A** shows G-RNA_1 to _4 (for G-RNA_2 to _4 the unique single-stranded guanosine gap have to be replaced by the corresponding ones). Panel **B** shows the A-RNA oligonucleotide, which was chosen as an uncleavable control. The oligonucleotide A-RNA_AG with a G:A mismatch is shown in Panel **C**. All secondary structures were predicted using the mfold 2.3 server of M. Zuker [http://mfold1.wustl.edu/~mfold/rna/form1.cgi, (7, 8)].

Digestion experiments with RNase T1. 50 pmol of the RNA samples were incubated with 1 µg (≈ 90 pmol) RNase T1 in 10 µl of 10 mM Tris/HCl buffer (pH = 7.0) for various time periods at 25 °C. The RNase T1 activity was stopped by the addition of 10 mM $ZnSO_4$. All samples were mixed with equal volumes of formamide and 10 µl of each sample were applied to a 12 % denaturing polyacrylamide gel. After a run of about 2 hours, the gel was stained with ethidium bromide and the bands visualized at 312 nm.

RESULTS AND DISCUSSION

We have designed various RNA oligonucleotides, with defined single-stranded gaps flanked by double-stranded regions. The series G-RNA_1 to G-RNA_4 is shown in FIG. 3A. The sequences were designed in a way, that no alternative secondary structures were suggested from the program mfold 2.3 [http://mfold1.wustl.edu/~mfold/rna/form1-2.3.cgi, (7, 8)] at folding temperatures up to 80 °C. Therefore the sequences should reliably fold as shown in FIG. 3. In the double-stranded helices additional mismatches were incorporated to prevent secondary structure formation during PCR.

In spite of the large self-complementary regions in the primers as well as in the templates high amounts of PCR products could be isolated after 30 cycles. 1 pmol of each DNA was used per T7 RNA polymerase transcription. The resulting RNAs were

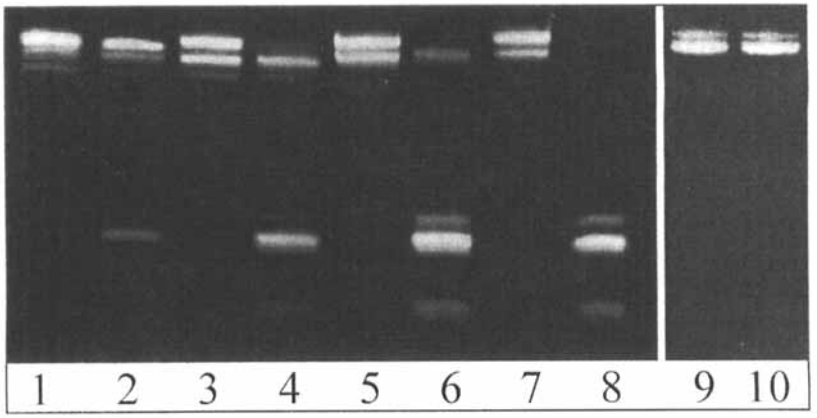


FIG. 4: Digestion of the oligonucleotides G-RNA_1 to _4 and A-RNA with RNase T1 separated on a denaturing 12 % polyacrylamide gel stained with ethidium bromide. Lanes 2, 4, 6 and 8 show the RNA-samples each after incubation with RNase T1 at 25 °C for 15 min (lane 2, G-RNA_1; 4, G-RNA_2; 6, G-RNA_3; 8, G-RNA_4). The RNA-samples after incubation in the cleavage buffer without RNase T1 are shown in lanes 1, 3, 5 and 7. Lane 9 and 10 show the controll A-RNA after incubation with (lane 10) and without (lane 9) RNase T1 in the cleavage buffer at 25 °C for 15 min.

denatured and renatured before using in the RNase T1 digestion assay. FIG. 4 shows the comparison of the cleavage products of RNA molecules with a gap of one, two, three or four nucleotides, respectively. The unpurified and undigested RNA-samples (lanes 1, 3, 5 and 7) show divergences in length and/or sequence based on the addition of nucleotides caused by an inaccuracy of T7 RNA polymerase, which is in agreement with published data (9). These divergences are partially visible in the RNase T1 digestion products (lanes 6 and 8), too.

Lane 2 shows, that RNase T1 is, although slowly, able to cleave within a gap of only one nucleotide. The turnover increased with the number of nucleotides in the gap (lanes 4, 6, 8). Although two cleavage products should be the result of RNase T1 digestion mainly one product band is visible in the gel. The second (smaller) product is only visible in the digestion products of the oligonucleotides G-RNA_3 and G-RNA_4 (lanes 6 and 8), but seems to be unstable after separation from the complete RNA. This might be due to partial unfolding of the two 3 bp long ds-regions and therefore additional cleavage points for RNase T1 could be generated. As a control an RNA-oligonucleotide with one

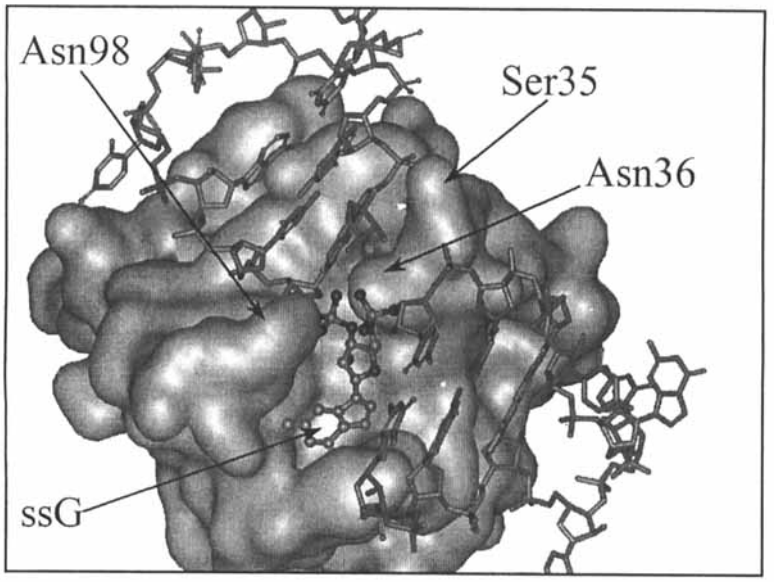


FIG. 5: Fitting of a one-guanosine gap RNA-oligonucleotide into the binding and active sites of RNase T1. An RNA-model of a unique single-stranded guanosine flanked by two three-base pairs stems and RNA-tetraloops were built applying the program MCSYM (11, 12). The guanosine was fitted into the binding and active sites with the previously obtained nucleic acid backbone conformation (5). The attached tetraloops both could be positioned without conflicts with the enzyme. Emphasized are the amino acids Ser35, Asn36 and Asn98 which hold the RNA-chain like fingers. Especially Ser35 and Asn36 could be involved in the binding of the guanosine part of a mismatch.

single-stranded adenosine in the gap was synthesized (FIG. 3B) and subjected to RNase T1 cleavage, too. No cleavage could be observed under identical conditions as for the various guanosine gaps (FIG. 4; lanes 9, 10).

The ability of RNase T1 to cleave inside a one nucleotide gap could be verified by considering previously obtained theoretical data (5), where we could determine an essential nucleic acid backbone conformation for a guanosine in a longer RNA-chain for a productive binding to RNase T1 (FIG. 1). Transferring these data to the guanosine in the one-nucleotide gap RNA-oligonucleotide, we could verify that the guanosine residue in this conformation could fit as well into the binding as into the catalytic site of RNase T1 without any conflicts between the two double-stranded regions and the enzyme. However, the guanosine has to flip outside of the RNA-chain leaving the possible

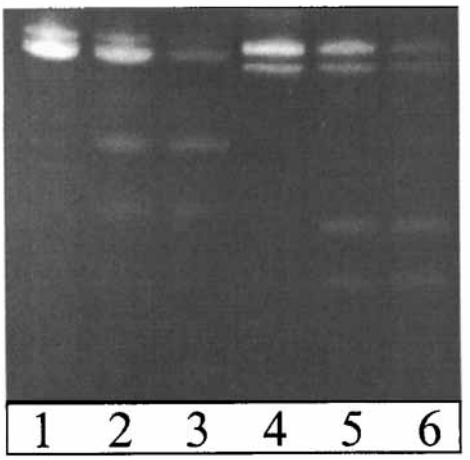


FIG. 6: Comparison of the cleavage reactions of a unique single-stranded guanosine in a gap and in a mismatch by RNase T1. Lanes 2 (A-RNA_AG) and 5 (G-RNA_1) show the RNA-samples after incubation with RNase T1 at 25 °C for 15 min and lanes 3 (A-RNA_AG) and 6 (G-RNA_1) after incubation for 60 min. Lanes 1 and 4 show the corresponding undigested oligonucleotides, respectively. The samples were separated on a denaturing 12 % polyacrylamide gel and stained with ethidium bromide.

stacking interactions with the continuing RNA-helices (FIG. 5). Due to this restriction the relative low turnover of RNase T1 concerning the G-RNA_1 RNA-oligonucleotide could be explained. The larger the single-stranded gap the larger is the flexibility and accessibility of the nucleotides and concomitantly the turnover increase as shown in FIG. 4.

We have also designed an RNA-oligonucleotide with one G:A mismatch inside the 3'-helix omitting any further single-stranded guanosine (FIG. 3C). This RNA was also subjected to RNase T1 digestion. Its comparison with the one-guanosine gap RNA-oligonucleotide is shown in FIG. 6. RNase T1 can cleave after the guanosine in the G:A mismatch, as well (lanes 2 and 3) and the turnover is comparable to that of the single-nucleotide gap (lanes 5 and 6). Whether the relatively slow cleavage next to the mismatch, compared to the larger gaps, could be explained by the necessity for the guanosine to flip out of the helix or by the probably restricted flexibility of the nucleic acid backbone in the mismatch, or by both, remains an open question. Also a conflict

between the amino acids Ser35 and Asn36 and the nucleic acid chain in opposite to the guanosine is possible (FIG. 5).

CONCLUSIONS

For the first time we herewith present a systematic investigation of the cleavage behavior of RNase T1 with respect to single-stranded gaps of varying sizes. We could demonstrate, that the enzyme is able to cleave even after only one single-stranded guanosine in a gap and next to a mismatch, as well. However, the turnover decreased strongly along with the length of the gap. Even the guanosine in a one-nucleotide gap can realize the necessary nucleic acid backbone conformation for a productive binding to RNase T1 without any conflicts with the flanking double-stranded regions and the enzyme.

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