Structure of secondary cleavage sites of \textit{E. coli} RNAaseIII in A3t RNA from bacteriophage T7

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Received November 14, 1986; Revised and Accepted December 17, 1986

ABSTRACT

Five 'secondary' cleavage sites of \textit{E. coli} RNAaseIII within the initiator RNA A3t (141 nucleotides) from bacteriophage T7 are described. Cleavage takes place apparently within or at the bottom of a short double-stranded stretch of RNA. Three secondary sites are efficiently cleaved in the presence of magnesium $^{2+}$ ions, two additional sites are cleaved in the presence of manganese $^{2+}$ ions at low monovalent salt concentrations (<0.05M).

INTRODUCTION

\textit{E. coli} RNAaseIII has been described as an endonuclease which cleaves double-stranded (ds) RNA molecules, reducing them to oligonucleotides averaging 15 bases in length creating new 5'-phosphate and 3'-OH ends. In \textit{vivo} this enzyme functions as a processing enzyme which cleaves single-stranded (ss) precursor RNA molecules at few active sites (1,2,3). Analysis of these sites in T7 mRNA (4,5,6) and \textit{E. coli} precursor rRNA (7,8) has revealed that these sites consist of an extended dsRNA region involving 20-30 base pairs of intramolecular base pairing. T7mRNA processing sites may contain a bubble of 6-9 unpaired bases in the dsRNA stem (6) and some sequence homology seems to be involved. Yet the significance of this sequence homology is unclear since other RNAaseIII processing sites seem to bear other sequence information (7,8,13). These processing sites in \textit{vivo} are identical to primary sites which are recognized by RNAaseIII in the presence of magnesium ($\text{Mg}^{2+}$) and a relatively high monovalent salt concentration (0.15 - 0.3 M) \textit{in vitro}. At lower monovalent salt concentration the stringency of cleavage by \textit{E. coli} RNAaseIII is lowered and additional sites are cleaved: the secondary sites.
The sites are not recognized in vivo by RNAaseIII. This property of RNAaseIII to trim RNA's depending of the monovalent salt concentration has been used successfully in vitro to analyse viral RNAs (10,11,12). However, the structure of the secondary sites has not been investigated in detail. Also it was unclear whether cleavage at one particular secondary site yields homogenous 5'- and 3'-ends or whether cleavage is unspecific in respect to the creation of new 5'- and 3'-ends at several positions. Here we show now that E.coli RNAaseIII introduces homogenous cuts in A3t RNA (141 bases) from bacteriophage T7 at relaxed (< 0.1 M) monovalent salt concentrations apparently at a relatively short stretch of base pairing in ssRNA. Additional secondary sites are cut in the presence of manganese (Mn2+) instead of magnesium (Mg2+) ions. RNAaseIII may therefore be used as a trimming tool for RNA molecules giving homogenous 5-ends.

MATERIALS AND METHODS
A3t RNA: T7 early mRNA was synthesized in vitro and digested with RNAaseIII under conditions where cleavage at primary sites takes place as described in ref. 9. The resulting products were separated on a 4% acrylamide gel and A3t RNA was eluted and precipitated twice before further use with two volumes of ethanol (100%) in the presence of carrier RNA (tRNA, 10μg/ml).

Labeled nucleotides
α- and γ-32P labeled nucleotides were purchased from ICN or NEN, respectively.

RNAaseIII was isolated from E.coli BL 15 (RNAasel-) (9).

RNA sequence analysis
Labeled A3t RNA was synthesized and isolated as described above and subjected to RNAase T1 and pancreatic RNAase fingerprinting analysis. RNAase T1 and pancreatic RNAase resistant oligonucleotides were subjected to further analysis and their sequence were determined by standard techniques (18). Further RNA sequence analysis was performed by nuclease P1 digestion and partial T1 and U2 digestion of 5'-end-labeled RNA's
followed by fingerprinting techniques or by sequence analysis on polyacrylamide gels.

Dephosphorylation and 5'-endlabeling of RNAs within the same reaction mixture was done as described in ref. 16. The determination of their sequence on gels was carried out as described in ref. 17.

Partial and complete nucleases P1 digestion was done as follows: 5'-endlabeled RNAs were incubated with 20 ng P1 enzyme (300 U/mg) in 10 μl volumina containing 0.05 M NH₄Ac pH 5.3 and 50 μg tRNA at room temperature. 3 μl aliquots were removed at 4, 10 and 20 minutes and added to 3 μl of 0.01 M EDTA at -80°C. The enzyme was inactivated by heating to 100°C for 4 minutes and then fingerprinted like described above. To determine the starting nucleotide at the 5'-end a complete digest of nuclease P1 was done with 10 μg P1 enzyme at 37°C for 1 hour. These digests were spotted on Whatman 3 MM paper and electrophoresed at 2000 Volts for 2 hours at pH 3.5. The starting nucleotide at the 5'-end was determined by comparison to the position of labeled markers.

RNAaseIII digestion
Was routinely performed in 100 μl volumina containing 0.02 M Tris-Cl pH 7.9, 0.005 M MgCl₂, 0.1 mM EDTA, 0.2 M NaCl, 0.1 mM DTT and 10 μg tRNA as carrier. After 20 minutes at 37°C 10 μl stopmix (2% SDS, 0.1 M EDTA) was added and then precipitated with two volumes of ethanol (100%) dried and dissolved in the desired sample buffer.

Polyacrylamide gel electrophoresis (PAGE)
was performed according to ref. 9.

RESULTS
As an approach to study secondary site cleavage by RNAaseIII of E.coli we used A3t RNA from bacteriophage T7 which originates at the RNA polymerase promoter site A3 (map position: 750; Ref. 6) and extends to the first RNAase III processing site (R 0.3) 141 nucleotides downstream. T7 early mRNA was synthesized with E.coli RNA polymerase in vitro and cleaved with RNAaseIII at primary sites. The A3t RNA was isolated after PAGE (Materials and Methods) and digested with
Figure 1: Effect of the NaCl concentration on the cleavage of RNAaseIII at secondary sites in A3t RNA in the presence of magnesium (5 mM).
RNAs were separated by PAGE (10-15% Acrylamide, Gelelektrophoresis conditions were according to ref. 9.)

RNAaseIII at various monovalent salt concentrations. Secondary sites in A3t RNA are observed in the presence of 0.005 M-0.05 M NaCl (Fig. 1). Higher concentrations of NaCl result in a resistance of A3t RNA to digestion by RNAaseIII. We located these sites in A3t RNA by sequencing the cleavage products.
Fingerprinting of RNAase T1 digested A3t RNA followed by comparison to identical treated RNA fragments after RNAaseIII digestion enabled us to localize the cleavage sites in oligonucleotides which were moving differently or were lacking compared to the complete A3t RNA digest (Fig. 2a). Sequence of the nucleotides was determined by standard techniques and compared to the sequence of ref. 6. Further analysis was performed with nuclease P1 digestion as well as partial RNAase T1 and U2 digestion of 5'-endlabeled RNA fragments.
5'-endlabeled A3t RNA was cut with RNAaseIII, (Fig. 2b, Material and Methods). The resulting bands A-H were extracted
Figure 2: Sequence analysis of RNAaseIII digested A3t RNA.
a) fingerprinting analysis of A3t RNA and RNA fragments after RNAaseIII digestion. Autoradiograph of RNAase T1 fingerprints. Arrows indicate some differences in the oligonucleotide pattern in comparison to the A3t RNA fingerprint. First dimension: electrophoresis; second dimension: homochromatography. 
b) 5'-end labeled RNA's after RNAaseIII digestion of A3t RNA separated by PAGE (8% acrylamide; 40 x 0.1 cm). c) Autoradiograph of fingerprinted partially nuclease P1 digested bands C,E,F. First dimension: electrophoresis; second dimension: homochromatography. Assignment of bases was in conjunction with the partial T1 and U2 cleavage data of Fig. 2d. The numbering system is according to the sequence in Fig. 3b. d) Partial RNAase T1 (G-specific) and RNAase U2 (A-specific) digest of bands B,C,E,G (5'-end labeled) analysed by PAGE (20% acrylamide).

from the gel, partially digested with nuclease P1 and fingerprinted. The sequence could be determined by the running spot analysis for every isolated fragment (Fig. 2c). To character-
Figure 3: Location of the secondary cleavage sites of RNAaseIII within the A3t RNA.

a) Secondary cleavage sites of RNAaseIII in A3t RNA and resulting RNA fragments. The intensity of the arrow indicates the efficiency of digestion by RNAaseIII. b) Computer constructed model of secondary structures (-21.7 kcal/Mol) in A3t RNA and the location of RNAaseIII secondary cleavage sites. The secondary structure was calculated according to ref. 19.

To resolve these bands further partial RNAase T1 and U2 digests were analysed on gels and compared to the sequence from ref. 6 (Fig. 2d). These analysis showed that cleavage of RNAaseIII at secondary sites yields homogenous 5' and 3' ends. The order of
the cleavage sites for A3t RNA is given in figure 3a. Band H was not considered further because it consisted of heterogeneous RNA classes. A computer constructed model of A3t RNA reflecting the most stable thermodynamic structure (-21.7 kcal/Mol) (19) shows that the secondary cleavage sites are on the bottom or in the middle of a ds stem region. The intensity of cleavage is: 1>2>3. A complete digestion of A3t RNA has not been obtained (Fig. 3a,b). This RNA configuration indicates that the intensity of cleavage could reflect the stability as well as the extension of dsRNA stems.

Furthermore we observed that RNAaseIII cleaves some ssRNAs differently in the presence of manganese (Mn$^{2+}$) instead of magnesium (Mg$^{2+}$) (Fig. 4a). Here we show that in a RNA molecule containing one primary RNAaseIII site (here: between A3t and 0.3 RNA) a different cleavage pattern occurs depending on the cation used (Mg$^{2+}$/Mn$^{2+}$). Primary sites are efficiently cleaved in the presence of Mg$^{2+}$ between 0.1 M - 0.3 M NaCl and in the presence of Mn$^{2+}$ from 0.3 M - 0.5 M NaCl. Secondary sites, however, are cleaved differently in the presence of Mn$^{2+}$ instead of Mg$^{2+}$ depending on the monovalent salt concentration. In 5'-endlabeled A3t RNA we localized two Mn$^{2+}$ dependent secondary sites (Fig. 4b). Their position as determined directly from the gel is also indicated in figure 3b. Therefore E.coli RNAaseIII cleavage in ssRNA can be influenced as well as by changing of monovalent salt concentration as by changing of divalent metal ions.

**DISCUSSION**

RNAaseIII has been isolated from different sources like Bacillus subtilis (13), Caulobacter crescentus (14) and Pseudomonas phaseolica (15). In all these cases RNAaseIII primary cleaves sites seem to involve extensive base pairing. Yet they all show a higher stringency towards processing (primary) sites also at relaxed low salt conditions than E.coli RNAaseIII (13,14). Cleavage of RNA at secondary sites therefore does not seem to be a common property of these enzymes.

Hence E.coli RNAaseIII offers an opportunity to cleave RNA to
various size classes just by relaxing the stringency of cleavage. These cleaved RNAs are, as we show here, homogenous at the 5'- and 3'-ends. Only a minor fraction (band H) contains nonhomogenous oligonucleotides.

The lowest stringency of cleavage seems to occur at low monovalent salt concentrations in the presence of Mn$^{2+}$, since in case of A3t RNA only in the presence of Mn$^{2+}$ efficient cleavage occurs after nucleotide 28 and 38. These sites are only cleaved inefficiently in the presence of Mg$^{2+}$ (Fig. 4b,3). Even if one acknowledges that the potential secondary structure of A3t RNA (Fig. 3b) has not been investigated by appropriate enzymes, this structure could indicate that RNAaseIII secondary sites may be located within or at the bottom of sequence involved in base pairing (Fig. 3b). These dsRNA stems are either very short (9 bases) or, if more extended, they are destabilized by mismatches. This feature seems the main difference to primary sites. A complete digestion of A3t RNA has not been obtained. It is obvious that the secondary structure of RNA refolds after cleavage at one particular site so that cleavage at other secondary sites is difficult or even impossible due to a new RNA configuration. So in vitro isolated band B,C,D,E could not be redigested efficiently (not shown).

As mentioned before RNAaseIII cleavage of secondary sites is not a common property of this type of enzymes. The E.coli

**Figure 4:** RNAaseIII digestion of RNA in the presence of divalent magnesium or manganese ions.

a) A3t + 0.3 RNA was isolated after PAGE as a partial digestion product of RNAaseIII of T7 early mRNA containing one primary site (between A3t and 0.3 RNA). RNAaseIII digestion products were analysed by PAGE (7-15% acrylamide). b) Manganese dependent cleavage of A3t RNA by RNAaseIII. Reaction products were analysed by PAGE (20% acrylamide). A3t RNA was incubated with RNAaseIII and the reaction products applied to an acrylamide gel (20%). 1. 5'-endlabeled A3t RNA cleaved by RNAaseIII in the presence of 0.02 M NaCl and 0.005 M MnCl$_2$; 2. homogenously labeled A3t RNA digested with RNAaseIII in the presence of 0.02 M NaCl and 0.005 M MnCl$_2$; 3. 5'-endlabeled A3t RNA digested with RNAaseIII in the presence of 0.02 M NaCl and 0.005 M MgCl$_2$; 4. alkaline treated 5'-endlabeled A3t RNA; 5. partially RNAase T1 digested 5'-endlabeled A3t RNA. Arrows indicate enhanced RNAaseIII cleavage of 5'-endlabeled A3t RNA in the presence of Mn$^{2+}$. The numbering system corresponds to the sequence in Fig. 3b).
RNAaseIII which is able to do so consists of two identical subunits (9). One possibility is that this enzyme in its dimeric form could recognize and cleave primary sites. In lowering the monovalent salt concentration RNAaseIII might dissociate and the monomers would then be able to cleave at some additional secondary structures in RNA. Alternatively mono- and divalent cations could modulate the affinity of the enzyme towards particular RNA structures, but more studies are needed to evaluate this behaviour of RNAaseIII of E.coli.

ACKNOWLEDGEMENT

G.G. was supported by a grant from the "Deutsche Forschungsgemeinschaft".

REFERENCES