Site specific enzymatic cleavage of RNA

Helen Donis-Keller

Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, MA 02138, USA

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ABSTRACT

The hybridization of a DNA oligonucleotide (a specific tetramer or longer) will direct a cleavage by RNase H (EC 3.1.4.34) to a specific site in RNA. The resulting fragments can then be labeled at their 5' or 3' ends, purified, and sequenced directly. This procedure is demonstrated with two RNA molecules of known sequence: 5.8S rRNA from yeast (158 nucleotides) and satellite tobacco necrosis virus (STNV) RNA (1240 nucleotides).

INTRODUCTION

Rapid, direct sequencing techniques exist for terminally labeled RNA molecules up to 200 nucleotides in length (1-6). However, the sequence determination of larger molecules still presents problems, since there are not methods to fragment RNA analogous to restriction endonuclease cleavage of DNA (7). Although one can isolate partial RNase cleavage products (8-10), it is laborious to prepare a sufficient quantity of large RNA fragments which can be ordered by sequence overlaps. This communication describes an enzymatic method which avoids this difficulty.

The method relies on the ability of ribonuclease H, purified from E. coli (11-13) or calf thymus (14-16), to attack only RNA-DNA hybrids breaking the RNA endonucleolytically to produce a 3' hydroxyl and a 5' phosphate at the point of cleavage (11,12,14-18). RNase H does not hydrolyze RNA-RNA hybrids, single-stranded RNA or DNA, or DNA-DNA hybrids (11,12,14,15,17-20). The principle of the method then is as follows: a synthetic DNA oligomer (e.g. a tetramer of known sequence) will hybridize to complementary single-stranded regions of an RNA molecule and direct cleavage by RNase H to those sites. DNA oligomers of different sequence will direct cleavage by RNase H to other regions of the RNA molecule. The resulting fragments can be labeled at the 5' or 3' ends, purified, and sequenced directly (1-6); the order of the fragments can be determined by overlaps in sequence or by mapping the
positions of the cuts in a fashion analogous to the restriction enzyme mapping of DNA (7,21).

MATERIALS AND METHODS

5.8S rRNA was isolated from phenol treated yeast cells (22) and purified by polyacrylamide gel electrophoresis (1). Dr. J. Stavrianopoulos kindly provided calf thymus RNase H. B. cereus RNase and DNA oligomers ATTACC and GCCG were given to me by Dr. J. Heckman and Dr. F. Kolpak respectively. Dr. J.M. Clark Jr. generously provided satellite tobacco necrosis virus RNA. RNase Phy M was isolated from the culture broth of Physarum polycephalum (unpublished results). Single-stranded DNA size markers were prepared by heat denaturing 5' end-labeled restriction fragments from the plasmid pBR 322 (23). The following were commercial products: [γ-32P]ATP and [5'-32P]pCp (New England Nuclear), T₄ RNA ligase (PL Biochemicals), calf intestinal phosphatase and T₄ polynucleotide kinase (Boehringer-Mannheim Biochemicals), synthetic DNA oligomers (Collaborative Research Inc.), E. coli RNase H (Enzo Biochem Inc.), and G-75 sephadex (Pharmacia Fine Chemicals Inc.).

5' and 3' End-Labeling

The 5' and 3' terminal phosphates were removed from the RNA with alkaline phosphatase (1) except that calf intestinal phosphatase in a buffer (50 μl) containing 0.1 M Tris-HCl pH 8.0, 1 mM MgCl₂, and 0.1 mM ZnCl₂ was used. The dephosphorylated RNA was 5' end-labeled with [γ-32P]ATP and T₄ polynucleotide kinase (1). 3' RNA termini were labeled with [5'-32P]pCp and RNA ligase (24). In a typical experiment 10 μg dephosphorylated STNV RNA (25 pMoles 3' ends) was added to a buffer containing 12.5 μM ATP (i.e. 5 x the concentration of [5'-32P]pCp), 50 mM HEPES pH 7.5, 20 mM MgCl₂, 3.3 mM DTT, 0.01 μg/μl BSA, and 10% DMSO. The mixture was added to 50 pMoles [5'-32P]pCp (lyophilized immediately prior to use). Sufficient T₄ RNA ligase was added to give a concentration of 5 μM enzyme and the mixture incubated for 12 hr at 4°C.

Hybridization of RNA to DNA Oligomers and Cleavage by RNase H

The conditions used for calf thymus RNase H and E. coli RNase H digests were adapted from those described by Stavrianopoulos (16) and Hurwitz (11) respectively. Each reaction mixture (10 μl) contained 0.1-2 μg terminally labeled RNA and 5 μg DNA oligomer in 50 mM Tris-HCl pH 8.3, 25 mM MgCl₂, 0.1 M KCl, 0.5 μg/μl BSA (calf thymus RNase H buffer), or 40 mM Tris-HCl pH 7.9,
4 mM MgCl₂, 1 mM DTT, 0.03 μg/μl BSA (E. coli RNase H buffer). In order to encourage hybridization the reaction mixtures were heated at 50°C for 3 min then for 30 min at 32°C. RNase H (0.2-1.5 units) was then added and the reaction allowed to proceed for 30 min at 32°C. 10 μl containing 10 M urea, 0.02% xylene cyanol and bromophenol blue was added and aliquots of 3-20 μl layered onto the 5-10% polyacrylamide, 7 M urea, 50 mM Tris-borate pH 8.3, 1 mM EDTA gels; electrophoresis buffer consisted of 50 mM Tris-borate pH 8.3, 1 mM EDTA. Alternatively, 10 μg carrier tRNA was added, the RNA precipitated with ethanol and dissolved in the urea dye mixture. For the preparation of larger amounts of RNA for sequencing purposes, 10 x the amount of RNA and 1 μg/μl DNA oligomer in 20 μl buffer was incubated for 1 hr at 32°C.

Removal of DNA Oligomer Prior to 5' or 3' End-labeling

Passage on G-75 sephadex separated the DNA oligomer from the RNA. A 3 ml polyethylene pipette tip plugged with silicated glass wool was filled with a slurry of 50% G-75 sephadex in 10 mM ammonium bicarbonate pH 8.0. After removing the buffer by a 1 min centrifugation in a table-top clinical centrifuge, the RNA-DNA sample, in 20 μl 10 mM ammonium bicarbonate, pH 8.0, was layered onto the column, and the RNA recovered by centrifugation for 30 seconds. Two washes, 20 μl and 50 μl of 10 mM ammonium bicarbonate pH 8.0 were then centrifuged through the column and combined with the RNA sample. There is a quantitative recovery of RNA molecules greater than 30 nucleotides in length. The RNA was recovered either by ethanol precipitation or lyophilization to remove the ammonium bicarbonate. The DNA oligomer can be quantitatively recovered by centrifuging 1 ml of 10 mM ammonium bicarbonate pH 8.0 through the column.

RNA Sequence Determination

Reaction conditions and buffer for partial enzymatic cleavage of 5' or 3' labeled RNA using RNase Phy M (U + A specific, unpublished results), T₁ (G specific), and U₂ (A specific) were as described (1) except that the reaction times were 10 min at 50°C and the reaction volume was 10 μl containing 0.2 μg/μl tRNA carrier; B. cereus partial digests (C + U specific, 4, and unpublished results) were carried out in the absence of urea.
RESULTS

Specific Cleavage of Yeast 5.8S rRNA

To determine whether RNase H could selectively break RNA at DNA oligomer-RNA hybrid structures, I hybridized a DNA hexamer (CGATGC or ATTACC) to 5' or 3' end-labeled 5.8S rRNA and incubated the mixture in the presence of calf thymus or E. coli RNase H (such hybridization mixtures will be referred to as RNA/d(oligomer)). Fig. 1 shows that calf thymus RNase H cleaved the RNA molecule specifically at regions complementary to the DNA hexamers producing a series of partial products whose lengths reflect the positions of the cutting points. This cleavage occurred only in the presence of both the DNA oligomer and RNase H; there was no cleavage when the RNA was incubated with either DNA oligomer or RNase H alone. The placement of the cutting points was determined by using the RNase T₁ and alkali patterns as markers, based on the known sequence of the 158 base long RNA (25). Longer runs (not shown) resolved interior portions of the molecule. The DNA oligomer CGATGC directed the calf thymus RNase H mainly to regions A, nucleotides 31-36, 6/6 bases paired; C, nucleotides 90-95, 5/6 bases paired; and D, nucleotides 153-158, 4/6 bases paired; however a light band is also apparent on the autoradiograph representing cleavage at region 15-20 (GGAUCU) (see lane 7, Fig. 1). Only one region, B, nucleotides 62-67, 5/6 bases paired, was cleaved using DNA oligomer ATTACC. This data suggests that as few as four paired bases provide a substrate for calf thymus RNase H.

Fig. 2 summarizes the results of an experiment that located the RNase H cleavage points more exactly. Fragments produced by preparative E. coli RNase H digestion of 5.8S rRNA/dCGATGC were labeled at their 5' or 3' ends,
Figure 2. 5.8S rRNA/dCGATGC partial cleavage fragments 5' or 3' end-labeled following digestion by E. coli RNaseH. The fragments are placed relative to the full length molecule. 5' end-labeled fragments (above sequence) and 3' end-labeled fragments (below sequence) were purified on a 20% polyacrylamide, 7 M urea, 50 mM Tris-borate pH 8.3, 1 mM EDTA, 1.5 mm thick gel. The sequence of each fragment was determined by comparison of the RNase T1 and limited alkaline hydrolysis patterns to the 5.8S rRNA known sequence (25). The amount of radioactivity found in each fragment is indicated by the thickness of the line representing the fragment.
purified by electrophoresis through a denaturing polyacrylamide gel, and identified by comparison of their RNase T1 and alkali patterns to the known sequence of 5.8S rRNA. Fig. 2 shows the placement of the fragments relative to the 5.8S rRNA molecule and regions of complementarity to the DNA oligomer CGATGC. The thickness of the arrows shows the relative recovery of the specific fragments found and identified.

It is clear from Figs. 1 and 2 that every break in the 5.8S rRNA is at an oligomer binding site. However, not every region of homology is cut by RNase H to a detectable extent. Neither the calf thymus or E. coli enzyme apparently cleaves the RNA at nucleotides 109-114 (AUAUG), nucleotides 123-128 (CGTACG), or at nucleotides 136-141 (CGTACG). These sites are located adjacent to or within a stable hairpin structure at nucleotides 116-137, observed by others (25,26), which may block cleavage by RNase H. Two other regions apparently uncleaved by calf thymus RNase H, nucleotides 24-29 (CGUUCG) and nucleotides 85-90 (GUAAAU), each contain only three contiguous base pairs and two internal mismatched bases.

The two enzymes differ in detail with regard to affinity for a particular hybrid structure. For example, while a hybrid region at nucleotides 43-48 (CCAGGC) is cleaved by the E. coli enzyme but the calf thymus enzyme must be fifty times more concentrated before any activity is apparent. The enzymes also differ in the position of cleavage at the hybrid site. Fig. 3 shows this difference at region A, nucleotides 31-36 (CCCATG). The calf thymus enzyme cuts the RNA at the phosphodiester bond between nucleotides U34 and C35, and at a secondary site between nucleotides C35 and G36, while the E.coli enzyme breaks the RNA mainly between nucleotides G36 and A37 under partial digestion conditions. A similar difference was observed at region C, nucleotides 90-95 (not shown). (Under the more complete digestion conditions used in the experiment shown in Fig. 2, secondary cuts inside or at the 3'
end of the hybrid region occurred; however, the dominant cuts are at the ends of the regions, usually the 5' end).

The difference in the mobility in Fig. 3 between the RNase H fragments and the alkali and RNase T₁ fragments is due to the terminal 3' hydroxyl groups on the RNase H fragments which retards their movement through the polyacrylamide gel relative to the RNase T₁ or alkali fragments which contain 3' phosphates. This assignment of the points of cleavage was confirmed by sequence analysis of fragments terminally labeled following 5.8S rRNA/dCGATGC cleavage by *E. coli* RNase H (Fig. 2).

**Specific Cleavage of STNV RNA**

Can one use RNase H/DNA oligomer cleavage to gain access to the interior of a large RNA molecule? I chose satellite tobacco necrosis virus (STNV) RNA, 1240 nucleotides in length, since its sequence was available; 42 bases at the 5' end (28) and 8 bases at the 3' end (29) have been directly determined, and recently W. Fiers and co-workers (30) have constructed and sequenced a DNA clone of a reverse transcript of STNV RNA which comprises all but 24 bases at the 5' terminus of the molecule. In order to determine which oligomers might produce cleavages within the interior of STNV RNA, I analyzed partial digests of end-labeled RNA with several DNA oligomers. Fig. 4 shows the cleavages produced by the *E. coli* and calf thymus RNase H's of the hybridized mixtures containing the DNA oligonucleotides ATTACC, C₆, ATGCAT, GCGC, CCGG, and 5' or 3' end-labeled STNV RNA. The oligomers directed RNase H to specific sites throughout the STNV molecule; the clarity of the pattern suggested that specific interior fragments suitable for sequencing might be obtainable.

**Figure 4.** Autoradiograph of calf thymus RNase H and *E. coli* RNase H cleavage products of 3' end-labeled STNV RNA (lanes 1-11) or 5' end-labeled STNV RNA (lanes 14-16) hybridized to DNA oligomers ATTACC, ATGCAT, C₆, GCGC, and CCGG. Calf thymus RNase H digests are represented by open circles adjacent to the lane number and *E. coli* RNase H digests by closed circles. Lanes 1 and 14, 1.5 units calf thymus RNase H (-) DNA oligomer; lanes 2, 5, 8, 15, and 16, 1.5 units calf thymus RNase H; lanes 3, 6, and 10, 0.5 units calf thymus RNase H; lanes 4, 7, and 11, 0.5 units *E. coli* RNase H; lane 9, 0.5 units *E. coli* RNase H (-) DNA oligomer; lane 12, 5' end-labeled pBR 322 Hind III and Bam/Pst restriction fragments heated at 90°C, 1 min in 10 M urea, 0.02% xylene cyanol and bromophenol blue, 20 mM EDTA prior to loading on the gel (5% polyacrylamide, 7 M urea, 50 mM Tris-borate pH 8.3, 1 mM EDTA, 1.5 mm x 33 cm x 40 cm); lane 13, Pst/Eco RI 5' end-labeled restriction fragments heat denatured to provide single-stranded DNA as in lane 12. The length of the DNA fragments is indicated in nucleotides (23). See text for details.
Are the Ends Produced by RNase H Suitable for Sequencing?

After cleaving unlabeled STNV RNA/dGCCGC with calf thymus RNase H, removing the oligomer, labeling the 5' end of the RNA fragments, and resolving them on two-dimensional polyacrylamide gels (D. Schwartz, personal communication), I sequenced the terminal 30-80 nucleotides of each fragment. Six fragments had 5' termini in the interior of the STNV RNA and seven fragments ran from the 5' terminus of the STNV RNA. The 5' ends of all six of the interior fragments were immediately preceded by GCCG sequences, according to the STNV sequence of Fiers and co-workers (30). Fig. 5 shows placement of the interior fragments. As an example, Fig. 6 shows the sequence of the 5' terminal 20 nucleotides of fragment B (Fig. 5). Longer electrophoresis resolved an additional 35 bases (not shown). This sequence occurs in the STNV RNA 983 nucleotides from the 5' end of the STNV RNA. The fragment continues to the 3' end of the molecule; since its length, 257 nucleotides, estimated from a logarithmic plot of nucleotide length versus distance from the origin using single-stranded DNA markers (23) as standards, corresponds to the predicted length. The first 55 bases of this RNA fragment agrees with the sequence found by Fiers and his co-workers (30) except at four positions: G1001, U1007, U1008, and A1010. The cloned STNV sequence has U1001, A1007, A1008, and U1010 at these positions. Also, the first base in the fragment could not be distinguished among the possible nucleotides A, C, or U. There are several possible explanations for these differences. The STNV RNA used here is strain SV-1, Rothamstead (31) while that of Fiers and his co-workers is strain SV-1, Uppsala (30). Moreover, the STNV RNA used in these experiments...
Figure 6. 5' terminal nucleotide sequence of STNV RNA fragment B (Fig. 5) (-) E lane, 5' end-labeled RNA incubated (-) RNase. Base specific partial cleavage of the RNA was obtained using RNase T1 (G), U2 (A), Phy M (U+A), and B. cereus (C+U). Limited alkaline hydrolysis provided cleavage at each base (OH). The reaction products were fractionated by electrophoresis through a 20% polyacrylamide, 7 M urea, 50 mM Tris-borate pH 8.3, 1 mM EDTA, 0.8 mm thick gel. See materials and methods for details.
experiments, isolated from a high passage stock of virus, consisted of a heterogeneous population of molecules (direct sequencing shows microheterogeneity, manuscript in preparation) and has sequence variations similar to those found with phage Qβ (32).

**Effects of RNA Secondary Structure**

Although the RNase H's cleave the RNA at regions of complementarity to the DNA oligomers, not all the primary sequence homologies are cut. The secondary structure of the RNA can prevent the formation of RNA-DNA hybrids or block their accessibility to RNase H. As mentioned before, in 5.8S rRNA a stable hairpin structure at nucleotides 116-137 (25,26) apparently blocks the cleavage by both RNase H's at three DNA oligomer binding sites. Furthermore, the two enzymes require buffers of different ionic strengths for optimal activity (11,16, the calf thymus RNase H reactions were done in a "high salt" buffer, 0.1 M KCl with 25 mM MgCl₂ and the *E. coli* RNase H digests in the absence of KCl, with 4 mM MgCl₂). The *E. coli* enzyme, active at a low salt concentration sees a greater range of sites than the calf thymus enzyme. Fig. 4 shows this difference with STNV RNA/dC₆; an additional break in the RNA is seen with the *E. coli* digest (see arrow, lane 11, Fig. 4) while cleavage at this location with calf thymus RNase H is negligible even with 3 times the amount of enzyme (lane 8, Fig. 4). It is possible that STNV RNA may have a more open structure in the "low salt" *E. coli* RNase H buffer.

**DISCUSSION**

These experiments show that RNase H specifically cleaves RNA hybridized to DNA tetramers or hexamers and that the RNA is cut within or immediately adjacent to the regions complementary to the DNA oligomers. Thus, different DNA oligomers provide a way to obtain a variety of cleavages at different locations within a large RNA molecule.

The RNase H/DNA oligomer cleavage method is analogous in some ways to the restriction endonuclease methods of mapping and fragmenting DNA molecules for sequence analysis (7,21). While a DNA endonuclease cleaves at sites containing the appropriate recognition sequence, RNase H cleaves at a "recognition structure". In most cases this "recognition structure" is a hybrid of at least 4 contiguous bases although other susceptible structures appear to be possible.

RNA secondary structure can block cleavage by RNase H in some places, nonetheless, internal RNA fragments that can be sequenced are produced using
the RNase H/ DNA oligomer procedure. The different cleavages directed by different DNA oligomers will overlap in sequence thereby ordering the fragments. This method is general and will serve in the sequence determination of any RNA molecule.

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