Base Cleavage Specificity of Angiogenin with *Saccharomyces cerevisiae* and *Escherichia coli* 5S RNAs†

Susanna M. Rybak and Bert L. Vallee*

Center for Biochemical and Biophysical Sciences and Medicine and Department of Pathology, Harvard Medical School and Brigham and Women's Hospital, Boston, Massachusetts 02115

Received September 21, 1987; Revised Manuscript Received December 1, 1987

ABSTRACT: The base cleavage specificity of angiogenin toward naturally occurring polyribonucleotides has been determined by using rapid RNA sequencing technology. With 5S RNAs from *Saccharomyces cerevisiae* and *Escherichia coli*, angiogenin cleaves phosphodiester bonds exclusively at cytidylic or uridylic residues, preferably when the pyrimidines are followed by adenine. However, not all of the existent pyrimidine bonds in the 5S RNAs are cleaved, likely owing to elements of structure in the substrate. Despite the high degree of sequence homology between angiogenin and ribonuclease A (RNase A), which includes all three catalytic as well as substrate binding residues, the cleavage patterns with natural RNAs are unique to each enzyme. Angiogenin significantly hydrolyzes certain bonds that are not appreciably attacked by RNase A and vice versa. The different cleavage specificities of angiogenin and RNase A may account for the fact that the former is angiogenic while the latter is not.

Recent reports from this laboratory have described the isolation, characterization, and cloning of angiogenin, a protein that induces neovascularization (Fett et al., 1985; Strydom et al., 1985; Kurachi et al., 1985). The primary sequence of angiogenin is highly homologous to that of the pancreatic ribonucleases (RNases),[1] and, indeed, angiogenin exhibits ribonucleolytic activity, albeit distinct from that of the degradative RNases (Shapiro et al., 1986). It does not display significant activity in standard RNase A assays with substrates such as C>p or poly(C), but it does catalyze the cleavage of isolated 28S and 18S rRNA.

Evidence is accumulating that an RNA substrate may be involved in the mechanism of angiogenin action (Shapiro et al., 1986; St. Clair et al., 1987). This and other physiologically important proteins that hydrolyze RNA (Gleich et al., 1986; Gullberg et al., 1986) may indeed represent a class of RNases with metabolic as contrasted with degradative or processing functions (Barnard, 1969). Although the primary target of angiogenin is still unknown, its characteristic activity toward naturally occurring polyribonucleotides has been recognized (Shapiro et al., 1986), and its base specificity with such substrates is of obvious importance. We have therefore examined the cleavage sites in 5S RNAs of known sequence to define the specificity and confirm the characteristic ribonucleolytic activity of angiogenin.

Materials and Methods

Materials. Angiogenin, free of any nonspecific ribonuclease, was purified from conditioned medium of a human colon adenocarcinoma cell line (HT-29) as described by Shapiro et al. (1986). Bovine pancreatic RNase A was purchased from Cooper Biomedical (Freehold, NJ). *Escherichia coli* 5S RNA and $tRNA^{Phe}$ were obtained from Boehringer Mannheim (Indianapolis, IN). Reovirus RNA was a gift from Dr. B. Fields. Dephosphorylated yeast 5S RNA, T_4 polynucleotide kinase, T_4 RNA ligase, and alkaline phosphatase were obtained from Bethesda Research Laboratories (Gaithersberg, MD). [γ-^{32}P]ATP (3000 Ci/mol) and [5′-^{32}P]pCp (3000 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Base-specific endonucleases were obtained in RNA sequencing kits from Pharmacia (Piscataway, NJ).

Preparation of ^{32}P-Labeled RNA Substrates. *E. coli* 5S RNA or $tRNA^{Phe}$ was dephosphorylated with alkaline phosphatase at 65 °C for 30 min and purified by electrophoresis on a 1.5-mm-thick polyacrylamide gel [10% acrylamide/0.6% *N*,*N*′-methylenebis(acrylamide)/7 M urea/50 mM Tris–borate (pH 8.3)/1 mM EDTA] for 12–15 h at 250 V. The RNA was located by UV shadow casting and the intact material excised. The gel was placed in siliconized, plugged, blue Eppendorf tips and crushed, and the RNA was eluted into 0.5 M ammonium acetate/1 mM EDTA by incubation at 37 °C for 6–12 h. The RNA was recovered by precipitation with ethanol. Dephosphorylated RNAs were labeled at the 5′ termini with [γ-^{32}P]ATP and T_4 polynucleotide kinase (Silberklang et al., 1979) and at the 3′ termini with [5′-^{32}P]pCp and RNA ligase as described by Peattie (1979). The ^{32}P-labeled RNAs were repurified as described above except that tRNA was added as a carrier in the final precipitation. RNA was suspended in autoclaved water and stored at −20 °C. All reagents and glassware were treated to destroy contaminating ribonucleases to ensure the integrity of the RNA.

Polyacrylamide Gel Analysis. ^{32}P-Labeled RNA (approximately 50000–100000 cpm/reaction) was digested with base-specific enzymes according to the protocol supplied by the manufacturer. For each experiment, the same amount (micrograms per milliliter) of ^{32}P-labeled RNA was digested with angiogenin or RNase A under nondenaturing conditions

† This work was supported by funds from Hoechst, A.G., under an agreement with Harvard University.

* Address correspondence to this author at the Center for Biochemical and Biophysical Sciences and Medicine, Seeley G. Mudd Building, 250 Longwood Ave., Boston, MA 02115.

[1] Abbreviations: RNase(s), ribonuclease(s); T_1, ribonuclease T_1; U_2, ribonuclease U_2; BC, ribonuclease *Bacillus cereus*; C>p, cytidine cyclic 2′,3′-phosphate; poly(C), poly(cytidylic acid); ATP, adenosine 5′-triphosphate; pCp, 3′-phosphocytidine 5′-phosphate; Py(s), pyrimidine(s); PyN, pyrimidine where N represents A, C, G, or U; XC, xylene cyanol; BPB, bromphenol blue; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

0006-2960/88/0427-2288$01.50/0 © 1988 American Chemical Society

in 25 mM Hepes, pH 7.2, 37 °C, for 15 min unless otherwise indicated. The reaction was stopped by addition of gel-loading buffer (10 M urea/1.5 mM EDTA/0.05% each of XC and BPB) and freezing on dry ice. A reference ladder was generated by alkaline hydrolysis with [^{32}P]RNA using 0.5 M sodium bicarbonate, pH 9. Five microliters from each reaction was electrophoresed through 10% acrylamide sequencing gels (0.4 mm thick, 40 cm long) at 40–50 W, initial voltage of about 1800 V. Low-voltage analysis was accomplished on 20 cm × 0.75 mm gels at 250 V. Under these conditions, XC comigrates with a 55-nucleotide fragment and BPB with a 12-nucleotide fragment. After electrophoresis, the gels were either covered with plastic wrap and directly exposed to Kodak XAR-5 film at −70 °C or soaked in 10% acetic acid/10% methanol and dried before exposure to the film. Autoradiograms were scanned in a Quick Scan R & D densitometer (Helena Laboratories, Beaumont, TX) or an LKB Ultrascan XL laser spectrophotometer.

Results

Base-Specific Cleavage Pattern of Angiogenin toward Natural Polyribonucleotides. Direct RNA sequence analysis (Donis-Keller et al., 1977; Simoncsits et al., 1977) of ^{32}P end-labeled 5S RNA from *Saccharomyces cerevisiae* or *E. coli* served to determine the sites of cleavage preference for angiogenin in a natural polyribonucleotide. 5S RNAs labeled at the 3′ or 5′ termini, respectively, present alternative cleavage points. Since the primary sequences of these RNAs are known (Barrell & Clark, 1974), the gel patterns generated by the base cleavage resulting from angiogenin can be compared with those of pancreatic and other ribonucleases to determine the specificity of angiogenin.

Limited digestion with angiogenin, 0.14–0.56 μM for 15 min at 37 °C, hydrolyzes only Py phosphodiester bonds as shown in autoradiograms of representative sequencing gels of 5S [3′-^{32}P]RNAs (Figures 1 and 2). Major angiogenin cleavage sites (arrows) in *S. cerevisiae* 5S RNA are identified between bases C_{113} and U_{62} (Figure 1) and between U_{103} and C_{62} in *E. coli* 5S RNA (Figure 2). In Figure 3a,b, heavy (major) and thin (minor) arrows have been employed to depict both major and minor cleavages, respectively, determined by sequencing both 3′- and 5′-labeled 5S RNA, in secondary structure models for *S. cerevisiae* (Figure 3A; Luehrsen & Fox, 1981) and *E. coli* (Figure 3B; Fox & Woese, 1975; Douthwaite & Garret, 1981). Since the amount of label accumulated at a particular site can vary for different limit digests, no attempt at quantitation was made. However, in order to indicate the degree of cleavage, arrows of different widths have been employed. The controls, i.e., undigested ^{32}P-labeled RNA in Figures 1 and 2, indicate some of the technical problems that interfere with quantitation. Thus, radiolytic damage generates bands in undigested controls, a phenomenon which repurification of the substrate does not alter. In *E. coli* (Figure 2), this occurs predominantly at U-A sites (Douthwaite & Garrett, 1981), and although U_{65}, U_{77}, and U_{103} appeared to be major angiogenin cleavage sites, corresponding bands in the undigested material put this in question. This also occurs at U_{90} in *S. cerevisiae* 5S RNA (Figure 1). Hence, we designate these cleavages as "weak" since angiogenin apparently merely enhances the base-line rate. This problem is not as evident with 5S RNA labeled at the 5′ end, since the specific activity of this labeled RNA is lower.

The assignments in Figure 3 are the consequence of at least three experiments for each labeled 5S RNA. They portray the cleavage generated by angiogenin from bases C_{10} to C_{113} in *S. cerevisiae* and from C_{11} to C_{110} in *E. coli* 5S RNA. Of

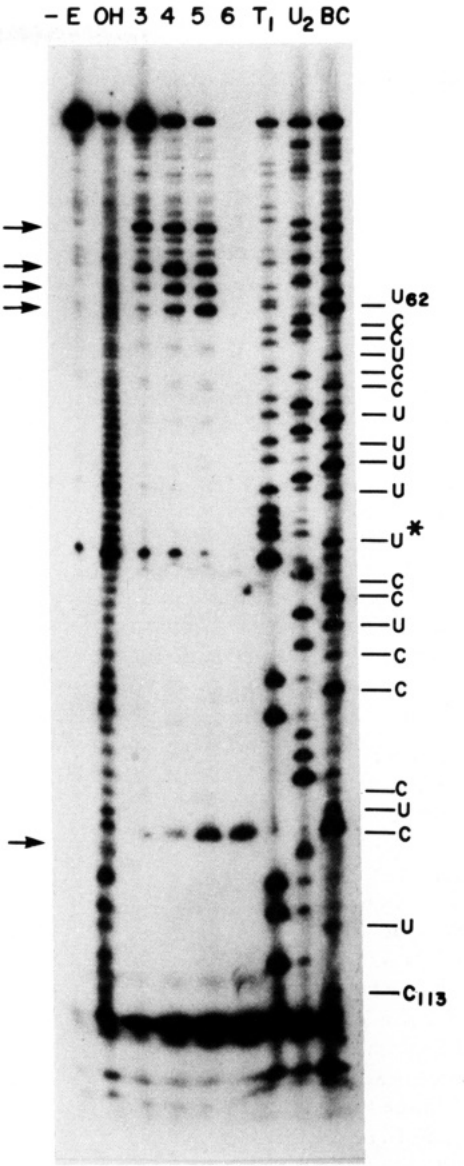

FIGURE 1: Cleavage pattern of angiogenin using *S. cerevisiae* 5S [3′-^{32}P]RNA as substrate. (Lanes 3, 4, 5, and 6) Cleavage of RNA (20 μg/mL) by 0.14, 0.28, 0.56, and 1.4 μM angiogenin, respectively, in a total volume of 5 μL. Hydrolysis was performed under nondenaturing conditions in 25 mM Hepes, pH 7.2 at 37 °C, for 15 min. The reactions were stopped and subjected to high-voltage polyacrylamide gel electrophoresis as described under Materials and Methods. (Lane –E) RNA incubated without angiogenin. (Lane OH) A reference ladder generated by alkaline hydrolysis as described under Materials and Methods. (Lanes T_1, U_2, and BC) Cleavage of RNA with RNase T_1, U_2, and BC, which cut preferentially after G, A, and Py's, respectively. One sequencing unit of each enzyme was used to obtain a partial digest of [^{32}P]RNA. Bases are numbered from the 5′ end of the RNA to the 3′ end according to the convention of Luehrsen and Fox (1981). Arrows represent major angiogenin cleavage sites. The ^{32}P-labeled RNA used in this experiment was gel purified twice, but fragments due to radiolytic damage persisted in the control (–E). The asterisk denotes bonds particularly sensitive to radiolytic damage.

the 16 major cleavage sites, 13 occur when A follows a Py. Further, it does not seem to matter whether C or U is the base preceding A. The fact that not all Py bonds are cleaved likely reflects the accessibility of the bond.

Relationship of Cleavage Pattern to Structure. Although the studies with angiogenin were performed under nondenaturing conditions, special care was not taken to reconstitute the native structure of the RNA (Douthwaite & Garret, 1981). The tertiary structure of these 5S RNAs is not known, and

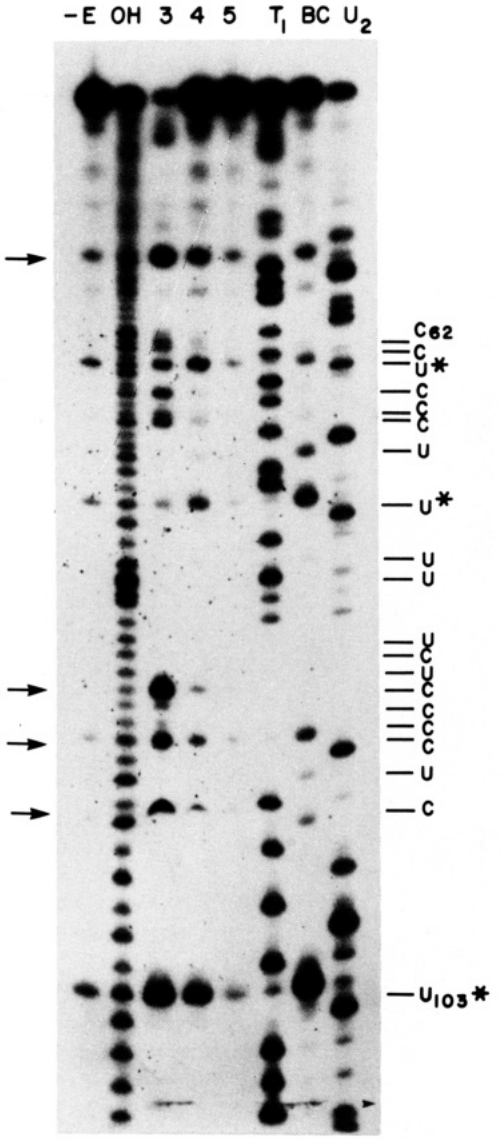

FIGURE 2: Cleavage pattern of angiogenin using *E. coli* 5S [3′-^{32}P]RNA as substrate. (Lanes 3, 4, and 5) Cleavage of RNA (200 μg/mL) by 0.56, 0.28, and 0.14 μM angiogenin, respectively. The asterisks denote bonds particularly sensitive to radiolytic damage, and the arrows represent major angiogenin cleavage sites. Other conditions are as in Figure 1 and under Materials and Methods except that the numbering of bases follows the convention of Fox and Woese (1975).

there is no universal agreement regarding their secondary structures (Chen & Marshall, 1986). Therefore, our attempts to correlate specific structural elements to cleavage points must be considered a preliminary indication that structure may be important to the ribonucleolytic specificity of angiogenin.

In *S. cerevisiae* 5S RNA, weak cleavage occurs in a long Py stretch from C_{28} to C_{40}. There is also weak cleavage in a comparable region of *E. coli* 5S RNA. This suggests that angiogenin does not cut well in Py-rich regions. In *E. coli* 5S RNA, C_{91}, C_{92}, and U_{95} also occur in a cluster of Py's close to very strong cleavages, which necessarily affects cleavage of adjacent bases (Douthwaite & Garret, 1981). In *S. cerevisiae*, there is a cluster of refractory Py's from C_{93} to C_{100} in a GC-rich helical region of the molecule, and in *E. coli*, refractory Py's occur from C_{19} to U_{32}, another GC-rich region. These constellations can cause base stacking which may render the bonds inaccessible to the enzyme (Peattie, 1979). U_{80} and U_{82} in *E. coli* occur in a region which causes compressions in the gel (Figure 2), indicating the occurrence of a "snap back" structure. U_{87}, C_{88}, and U_{89} are resistant to ribonuclease when

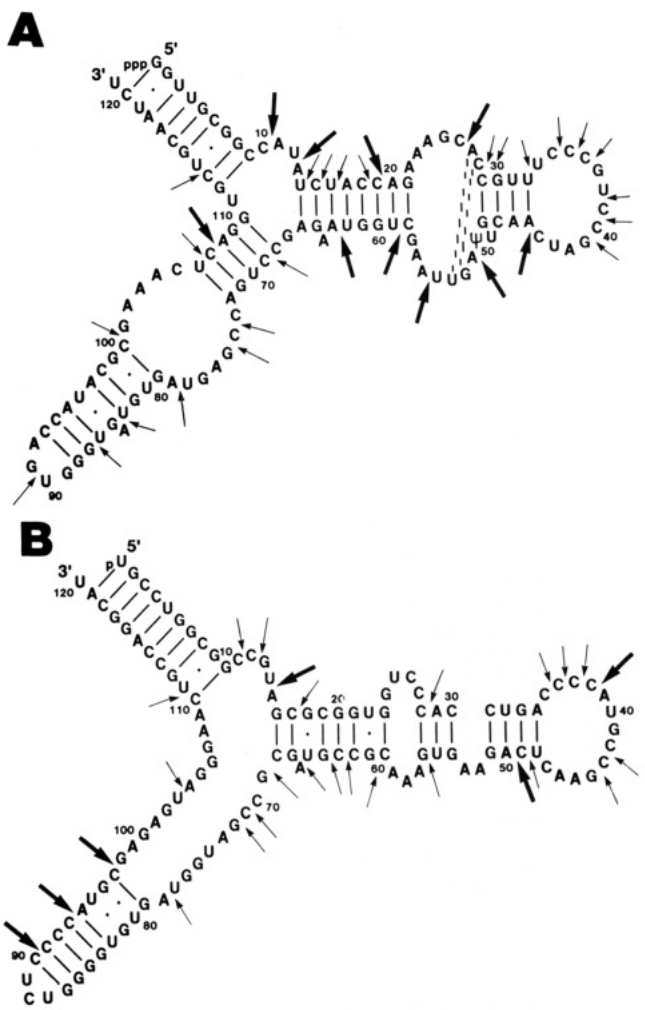

FIGURE 3: Secondary structural model of (A) *S. cerevisiae* 5S RNA drawn according to Luehrsen and Fox (1981) and (B) *E. coli* 5S RNA according to Fox and Woese (1975) with modifications by Douthwaite and Garrett (1981). The arrows mark the cleavage sites of angiogenin as determined by at least three sequencing experiments of both 5′- and 3′-labeled 5S RNA. The heavy arrows correspond to major cleavage sites and the thin arrows to minor cleavage sites.

E. coli 5S RNA is in the low-salt form (Christensen et al., 1985) as would likely be the case in the digestion buffer used in these studies.

E. coli $tRNA^{Phe}$ was also subjected to RNA sequence analysis after digestion by angiogenin. The results are entirely consistent with the present data and establish the cleavage preference of angiogenin on naturally occurring polyribonucleotides to be Py specific with major cleavages occurring mainly after PyA bonds. The susceptibility of PyN bonds to angiogenin depends on their position in the molecule relative to structure or neighboring bases, i.e., Py stretches.

Hydrolysis of Naturally Occurring Polyribonucleotides by Angiogenin. The ^{32}P-labeled fragments generated by angiogenin digestion of yeast 5S RNA (Figure 4) and $tRNA^{Phe}$ (Figure 5) were analyzed by low-voltage urea–polyacrylamide gel electrophoresis. Since the latter has a more highly ordered structure than the former (Tewari & Burma, 1982), a comparison of the gel patterns should indicate the extent to which RNA structure may influence the ribonucleolytic activity of angiogenin. Equal quantities of RNA (25 μg/mL) were digested with angiogenin, and the fragments generated in each case were analyzed. Those fragments containing a ^{32}P label were visualized by autoradiography. Their sizes, estimated from a standard curve, determine the distance from the cleavage site to the labeled end of the molecule.

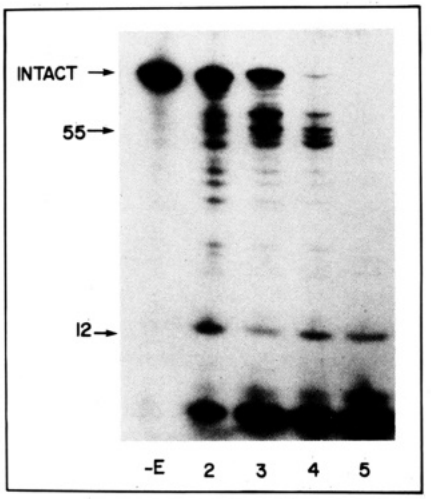

FIGURE 4: Fragments of *S. cerevisiae* 5S [3′-^{32}P]RNA separated by low-voltage electrophoresis. (Lanes 2, 3, 4, and 5) Cleavage of RNA (25 μg/mL) by 0.14, 0.28, 0.56, and 1.7 μM angiogenin, respectively. The digestion conditions correspond to those described in the legend to Figure 1 and under Materials and Methods. (Lane −E) RNA incubated without angiogenin. The positions of XC (55; nucleotides) and BPB (12; nucleotides) are indicated. The identification of the fragments is deduced from the sequencing gels after determining approximate sizes from a standard plot according to the migration of the intact material and the two dyes. The autoradiogram was scanned with the R & D Quick Scan densitometer.

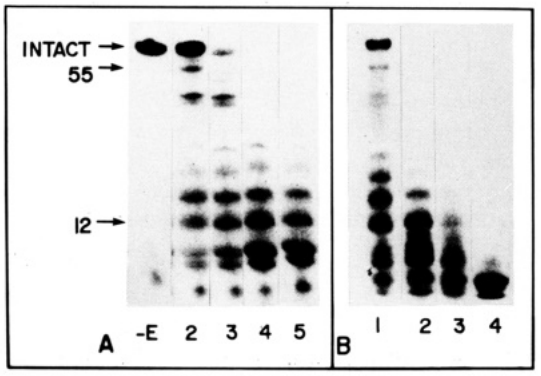

FIGURE 5: Fragments of *E. coli* [5′-^{32}P]tRNA^{Phe} separated by low-voltage electrophoresis. (A) (Lanes 2, 3, 4, and 5) Cleavage of tRNA (25 μg/mL) by 0.14, 0.42, 1.71, and 3.43 μM angiogenin, respectively. The digestion conditions correspond to those described in the legend to Figure 1 and under Materials and Methods. (Lane −E) RNA incubated without angiogenin. Assignment of fragment sizes is described in the legend to Figure 4. (B) (Lanes 1, 2, and 3) Cleavage of tRNA by 0.28, 8.50, and 17.10 μM angiogenin, respectively. (Lane 4) Digestion of RNA by 0.28 μM RNase A.

Increasing concentrations of angiogenin progressively degrade intact 5S RNA until a limit digest is reached (Figure 4). With 1.7 μM angiogenin, 80% of the total oligonucleotide product appears in fragments of from five to eight bases (Figure 4, lane 5). The remaining 20% appears in a 14-nucleotide fragment which resists further digestion by up to 8.5 μM angiogenin (not shown). This fragment, which derives from the 3′ end of the molecule by cleavage at C_{107}, may fold back on itself by complementary base pairing to form double-stranded RNA which resists digestion. Angiogenin is known to prefer single-stranded RNA as a substrate (Shapiro et al., 1986). Alternatively, the fragment may be double stranded by remaining hybridized to its complementary RNA at the 5′ end of the 5S RNA, since a cleavage site is also located 10–12 nucleotides from the 5′ end of the molecule.

The cleavage pattern obtained on digestion of *E. coli* tRNA^{Phe} with angiogenin is shown in Figure 5A. Angiogenin

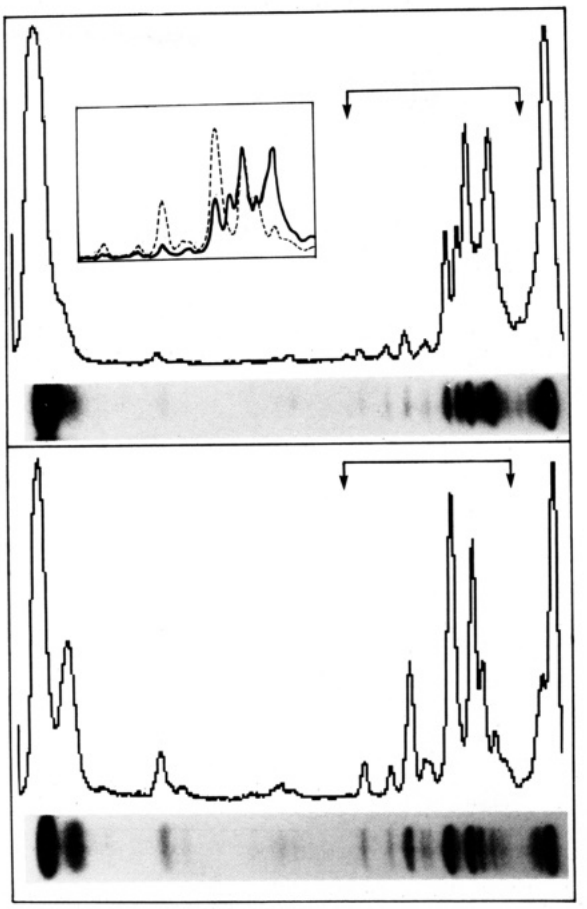

FIGURE 6: Comparison of fragments of *S. cerevisiae* 5S [3′-^{32}P]RNA generated by angiogenin (top panel) or RNase A (bottom panel). The arrows denote regions of the figure which are superimposed (insert, top panel; angiogenin, solid line; RNase A, dashed line). The RNA preparation, digestion, and electrophoresis are described in the legend to Figure 4 and under Materials and Methods. Concentrations of angiogenin and RNase A which generated comparable partial digests are 2.8×10^{-1} μM and 1.4×10^{-4} μM, respectively. The autoradiograph was scanned with the LKB Ultrascan XL laser spectrophotometer. The gel origin is to the right.

generates fragments that range in size from 5 to 25 nucleotides which are quite resistant to further digestion with concentrations up to 17 μM angiogenin (Figure 5B). In contrast, 0.28 μM RNase A digests tRNA^{Phe} to fragments smaller than pentanucleotides (Figure 5B, lane 4). The angiogenin-resistant fragments derive from a GC-rich region of tRNA^{Phe}, and this resistance most likely reflects the stability of specific tertiary structure regions within the tRNA since other regions of the tRNA do undergo significant cleavage.

Comparison of Cleavage Patterns of 5S RNA Generated by Angiogenin or RNase A. Angiogenin and RNase A generate completely different cleavage patterns from yeast 5S RNA (Figure 6). A scan of the autoradiograph readily reveals both qualitative and quantitative differences. Figure 7 is a high-voltage separation of the same labeled RNA fragments, and Py's cleaved by angiogenin are indicated. Although regions of the 5S RNA are inaccessible to hydrolysis by either enzyme, these regions are not identical. Thus, RNase A generates strong cleavages in a region from U_{86} to C_{68}, where angiogenin is barely active. Conversely, angiogenin makes a strong cut after C_{58}, indicated by an arrow in Figure 7.[2] In contrast, RNase A does not cleave this bond as well. Ang-

[2] Although C_{58} and U_{59} are not distinguished in the 3′-labeled 5S RNA shown in Figure 7, the identification of $C_{58}U$ as the bond cleaved is clearly evident with 5′-labeled molecules.

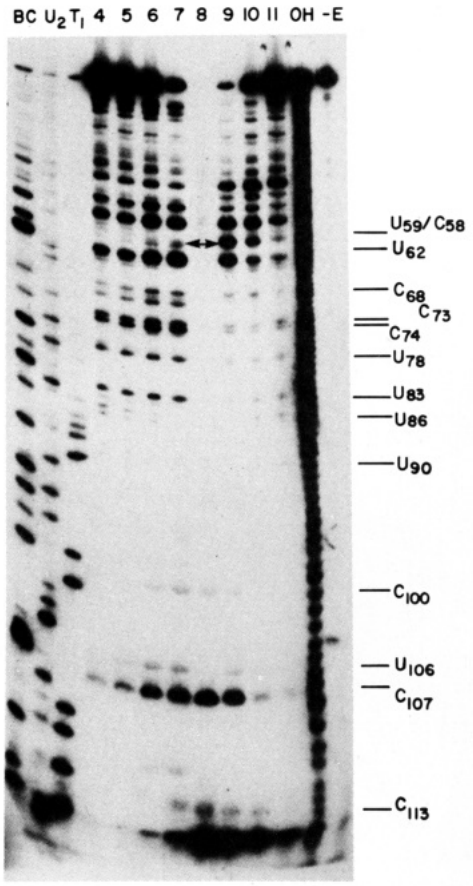

FIGURE 7: High-voltage electrophoresis of fragments of *S. cerevisiae* 5S [3′-^{32}P]RNA generated by angiogenin or RNase A. The RNA preparation is described in the legend to Figure 4. Digestion and electrophoresis are described in the legend to Figure 1 and under Materials and Methods. (Lanes 4, 5, 6, and 7) Digestion of 5S RNA by 2.8×10^{-6}, 2.8×10^{-5}, 2.8×10^{-4}, and 1.4×10^{-4} μM RNase A, respectively. (Lanes 8, 9, 10, and 11) Digestion of 5S RNA by 1.4, 0.56, 0.28, and 0.14 μM angiogenin, respectively. (Lane –E) RNA incubated without enzyme; (lane OH) an RNA ladder generated by alkaline hydrolysis; (lanes BC, U_2, and T_1) digestion by base-specific (Py's, A, and G, respectively) ribonucleases. Py's cleaved by angiogenin are indicated to the right of the gel. The arrow indicates a bond preferentially cleaved by angiogenin.

iogenin strongly cleaves C_{90} in *E. coli* 5S RNA, a bond affected insignificantly by RNase A under standard digestion conditions at 37 °C (Figure 8A). The amount of label in a particular band can also vary with the extent of the limit digest. Therefore, to ensure equal partial digests and confirm the strong cleavage of C_{90} by angiogenin, limited digests of *E. coli* 5S RNA by angiogenin or RNase A were performed at 0 °C. The results in Figure 8B demonstrate that angiogenin indeed cleaves C_{90} but RNase A does not.

Hydrolytic Activity of Angiogenin toward Other Nucleic Acids. Since some nucleases hydrolyze both RNA and DNA (Barnard, 1969), the cleavage activity of angiogenin toward other naturally occurring nucleic acids was examined under the conditions used in these studies to digest 5S RNA. Double-stranded reovirus RNA is not degraded by concentrations of angiogenin that digest 5S RNA, although some degradation was evident at higher concentrations, i.e., 14 μM.

The nucleolytic activity of angiogenin toward single-stranded DNA was tested by using the plus strand of M13mp19 viral DNA. Cleavage was not observed, even with higher concentrations of angiogenin. Interestingly, angiogenin appears to bind both to single-stranded DNA and to double-stranded plasmid or high molecular weight DNA, as will be reported in detail.

Discussion

Recent studies of the specificities of RNases have been facilitated by the application of rapid RNA sequencing technology (Smith et al., 1981; Wreschner et al., 1981; Boguski et al., 1980; Saha, 1982). Using an RNA molecule of known sequence, RNase specificity may be determined by comparing a partial digest of this substrate by the enzyme under examination with partial digests derived from RNases of known base preference. The fact that angiogenin does not exhibit activity toward standard RNase A substrates (Shapiro et al., 1986) called for the determination of the cleavage pattern of angiogenin on natural polyribonucleotides, hence the approach in the present work. Further, the specific activity of angiogenin toward natural RNAs differs from that of RNase A (Shapiro et al., 1986), indicating differences in the sites and/or nature of the cleavages of the two enzymes.

The present study demonstrates that angiogenin cleaves a natural RNA exclusively after pyrimidines, most frequently when the pyrimidine is followed by adenine. Therefore, the difference in cleavage specificity between angiogenin and RNase A is not due to base preference since both cleave only after pyrimidines. Actually, this is not surprising since the catalytic residues and those that constitute the substrate binding region are all highly conserved in angiogenin (Strydom et al., 1985).

We have no evidence thus far that cleavage by angiogenin requires a specific recognition element in terms of the primary nucleotide sequence of 5S RNAs. Therefore, elements of RNA structure likely confer specificity consistent with the properties of some processing endonucleases (Stahl et al., 1980; Eichler & Eales, 1983).

Any correlation of angiogenin cleavage sites with specific structural elements can only be interpreted in general terms. No efforts were made to reconstitute the native structure of 5S RNAs or to determine primary cleavage sites by angiogenin precisely. Nevertheless, under the conditions of moderate ionic strength and low temperature employed here, 60–70% of the nucleotide residues in 5S RNAs would be expected to be involved in base pairing (Attardi & Amaldi, 1970). Therefore, it is reasonable to assume that the restricted cleavage specificity, i.e., the failure of all PyN bonds to be cleaved by angiogenin, is a consequence of the imperviousness of certain regions to attack imposed by their secondary structure.[3] The effects of structure are also evident in the low-voltage analysis of limit digests, since regions in 5S RNA and tRNA that are not digested easily derive from areas of the RNAs presumed to form stable, base-paired structures.

Interestingly, two major cleavage sites after C_{90} in *E. coli* 5S RNA and C_{58} in *S. cerevisiae* 5S RNA, respectively, are not followed by adenine and are not cleaved efficiently by RNase A. Both pyrimidines are in putative double-stranded regions of the molecule, and both enzymes prefer single-stranded RNA as substrate. This strongly implies that some element of structure is crucial to cleavage specificity by angiogenin. The possibility exists that after binding to a specific site on RNA angiogenin could alter the local conformation to induce susceptibility to cleavage.

These views are supported by recent work from this laboratory which describes a limited, selective cleavage of rabbit reticulocyte ribosomes by angiogenin as opposed to the random nicking engendered by RNase A (St. Clair et al., 1987).

[3] Under other conditions of pH and temperature used to minimize effects of RNA structure, angiogenin cleavage sites were enhanced and more evenly distributed.

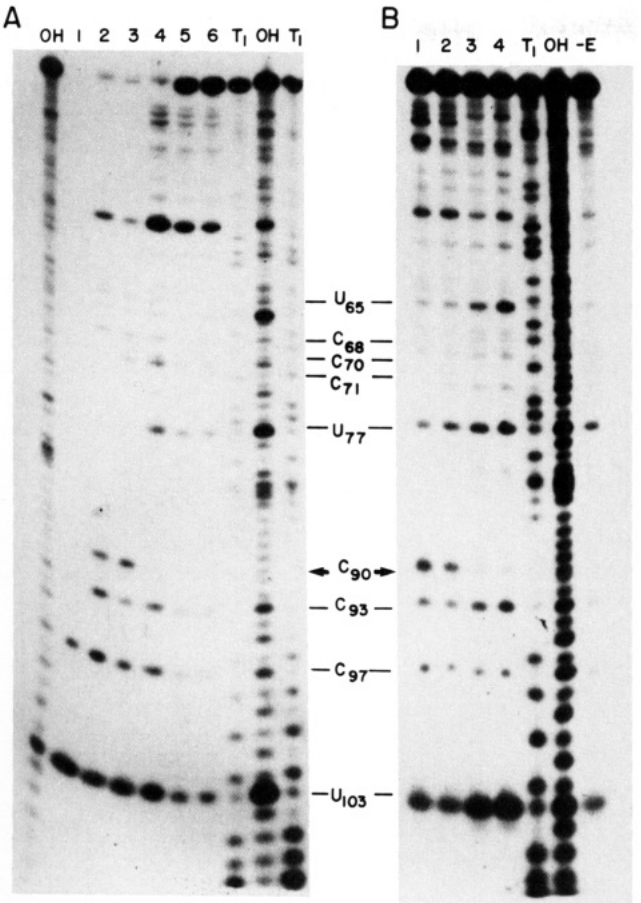

FIGURE 8: High-voltage electrophoresis of fragments of *E. coli* 5S [3′-^{32}P]RNA generated by angiogenin or RNase A. (A) (Lanes 1, 2, and 3) 5S RNA (10 μg/mL) digested at 37 °C with 1.4, 0.56, or 0.28 μM angiogenin, respectively; or (lanes 4, 5, and 6) with 3.3×10^{-4}, 1.6×10^{-5}, or 8.3×10^{-6} μM RNase A, respectively, under conditions described in the legend to Figure 1 and under Materials and Methods. (B) (Lanes 1 and 2) 5S RNA was digested at 0 °C with 0.28 μM angiogenin for 15 min or 2 h, respectively, or (lanes 3 and 4) with 3.3×10^{-4} μM RNase A for 15 min or 2 h, respectively. Other symbols as in Figure 7.

Cleavage of the ribosome by angiogenin completely inhibits protein synthesis when ribosomes are translated in an in vitro reticulocyte lysate system. The results of the present study support the idea that the intricate architecture of rRNA in the ribosome can present a cleavage site(s) specifically recognized by angiogenin.

While the exact specificity and precise substrate as well as the functional implications of angiogenin's enzymatic activity, with regard to its physiological potential, remain to be determined, this study establishes some features of the sites where angiogenin cleaves in a natural polyribonucleotide. There is compelling evidence that some form of RNA will turn out to be the specific substrate for angiogenin since its ribonucleolytic activity correlates with its angiogenic activity (Shapiro et al., 1986). The angiogenin gene codes for a leader sequence, suggesting that this protein is a secreted enzyme (Kurachi et al., 1985), consistent with its presence in human plasma (Shapiro et al., 1987). However, there is no information as yet regarding either the nature or the locale of a putative, native substrate. Small nuclear RNAs, naturally occurring antisense RNAs (Haywood, 1986), and yet other oligonucleotides (Plesner et al., 1987) have not been ruled out as possible substrates. Further, angiogenin also is a potent inhibitor of reticulocyte ribosomal function (St. Clair et al., 1987). The mechanism of the interaction of angiogenin with cellular and/or extracellular RNAs and the identification of an angiogenin target promise to elucidate a new pathway for the induction and regulation of neovascularization.

ACKNOWLEDGMENTS

We thank Drs. Stanislawa Weremowicz and Robert Shapiro for specially purified angiogenin and Yvette Rheault for excellent technical assistance.

Registry No. RNase, 9001-99-4.

REFERENCES

Attardi, G., & Amaldi, F. (1970) *Annu. Rev. Biochem. 39*, 183–219.

Barnard, F. A. (1969) *Annu. Rev. Biochem. 38*, 677–732.

Barrell, B. G., & Clark, B. F. C. (1974) in *Handbook of Nucleic Acid Sequences*, p 68, Joynson-Bruvvers, Oxford, England.

Boguski, M. S., Hieter, P. A., & Levy, C. C. (1980) *J. Biol. Chem. 255*, 2160–2163.

Chen, S., & Marshall, A. G. (1986) *Biochemistry 25*, 5117–5125.

Christensen, A., Mathiesen, M., Peattie, G., & Garrett, R. A. (1985) *Biochemistry 24*, 2284–2291.

Donis-Keller, H., Maxam, A. M., & Gilbert, W. (1977) *Nucleic Acids Res. 4*, 2527–2538.

Douthwaite, S., & Garrett, R. A. (1981) *Biochemistry 20*, 7301–7307.

Eichler, D. C., & Eales, S. J. (1983) *J. Biol. Chem. 258*, 10044–10053.

Fett, J. W., Strydom, D. J., Lobb, R. R., Alderman, E. M., Bethune, J. L., Riordan, J. F., & Vallee, B. L. (1985) *Biochemistry 24*, 5480–5486.

Folkman, J., & Klagsbrun, M. (1987) *Science (Washington, D.C.) 235*, 442–447.

Fox, G. E., & Woese, C. (1975) *Nature (London) 256*, 505–507.

Gleich, G. J., Loegering, D. A., Bell, M. P., Checkel, J. L., Ackerman, S. J., & McKean, D. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3146–3150.

Gullberg, U., Widegren, B., Arnason, U., Egesten, A., & Olsson, I. (1986) *Biochem. Biophys. Res. Commun. 139*, 1239–1242.

Haywood, S. M. (1986) *Nucleic Acids Res. 14*, 6771–6772.

Kurachi, K., Davie, E. W., Strydom, D. J., Riordan, J. F., & Vallee, B. L. (1985) *Biochemistry 24*, 5494–5499.

Luehrsen, K. R., & Fox, G. E. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2150–2154.

Peattie, D. A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1760–1764.

Plesner, P., Goodchild, J., Kalckar, H. M., & Zamecnic, P. C. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1936–1939.

Saha, B. K. (1982) *Nucleic Acids Res. 10*, 645–652.

Shapiro, R., Riordan, J. F., & Vallee, B. L. (1986) *Biochemistry 25*, 3527–3532.

Shapiro, R., Strydom, D. J., Olson, K., & Vallee, B. L. (1987) *Biochemistry 26*, 5141–5146.

Silberklang, M., Gillum, A. M., & RajBhandary, U. L. (1979) *Methods Enzymol. 59G*, 58–109.

Simoncsits, A., Brownlee, G. G., Brown, R. S., Rubin, J. R., & Guilley, H. (1977) *Nature (London) 269*, 833–836.

Smith, G. F., Slattery, E., & Lengyel, P. (1981) *Science (Washington, D.C.) 212*, 1030–1032.

Stahl, D. A., Meyhack, B., & Pace, N. R. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5644–5648.

St. Clair, D. K., Rybak, S. M., Riordan, J. F., & Vallee, B.

L. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8330–8334.
Strydom, D. J., Fett, J. W., Lobb, R. R., Alderman, E. M., Bethune, J. L., Riordan, J. F., & Vallee, B. L. (1985) *Biochemistry 24*, 5486–5494.
Tewari, D. S., & Burma, D. P. (1982) *Biochem. Biophys. Res. Commun. 109*, 256–261.
Wreschner, D. H., McCauley, J. W., Skehel, J. J., & Kerr, I. M. (1981) *Nature (London) 289*, 414–417.

Steric Course of the Hydration of D-*gluco*-Octenitol Catalyzed by α-Glucosidases and by Trehalase†

Wolfgang Weiser and Jochen Lehmann
Chemisches Laboratorium, Universität Freiburg, D-7800 Freiburg i Br, West Germany
Seiya Chiba,‡,§ Hirokazu Matsui,‡,§ Curtis F. Brewer,‖ and Edward J. Hehre*,‡
Department of Microbiology and Immunology and Department of Molecular Pharmacology, Atran Foundation Laboratory, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461
Received October 20, 1987; Revised Manuscript Received December 11, 1987

ABSTRACT: Crystalline *Aspergillus niger* α-glucosidase and highly purified preparations of rice α-glucosidase II and *Trichoderma reesei* trehalase were found to catalyze the hydration of [2-^{2}H]-D-*gluco*-octenitol, i.e., (*Z*)-3,7-anhydro-1,2-dideoxy-[2-^{2}H]-D-*gluco*-oct-2-enitol, to yield 1,2-dideoxy-[2-^{2}H]-D-*gluco*-octulose. In each case, the stereochemistry of the reaction was elucidated by examining the newly formed centers of asymmetry at C-2 and C-3 of the hydration product. The C-1 to C-3 fragment of each isolated [2-^{2}H]-D-*gluco*-octulose product was recovered as [2-^{2}H]propionic acid and identified by its positive optical rotatory dispersion as the *S* isomer, showing that each enzyme had protonated the octenitol (at C-2) from above its *re* face. ^{1}H NMR spectra of enzyme/D-*gluco*-octenitol digests in D_2O showed that the α-anomer of [2-^{2}H]-D-*gluco*-octulose was exclusively produced by each α-glucosidase, whereas the β-anomer was formed by action of the trehalase. The trans hydration catalyzed by the α-glucosidases was found to be very strongly inhibited by the substrate; the cis hydration reaction catalyzed by the trehalase showed no such inhibition. Special importance is attached to the finding that in hydrating octenitol each enzyme creates a product of the same anomeric form as in hydrolyzing an α-D-glucosidic substrate. This result adds substantially to the growing evidence that individual glycosylases create the configuration of their reaction products by a means that is independent of donor substrate configuration, that is, by a means other than "retaining" or "inverting" substrate configuration.

In recent years much new insight into the catalytic capabilities of glycosidaes and glycosyltransferases has been obtained through the study of reactions catalyzed without glycosidic bond cleavage. Studies with glycosyl fluorides and enolic glycosyl donors have, for example, provided several converging lines of evidence for the ability of individual glycosylases to promote different stereochemical reactions. Enzymes that hydrolyze glycosidic substrates with configurational inversion have been found to hydrolyze the corresponding anomer of a glycosyl fluoride but also to catalyze stereocomplementary nonhydrolytic reactions with the other anomer (Hehre et al., 1979, 1982; Kitahata et al., 1980; Kasumi et al., 1986, 1987). Glycosidases and certain glucanases (i.e., β-amylase and cellulases) that catalyze glycal hydration have been shown to protonate that type of substrate from a direction opposite that generally assumed for their glycosidic substrates (Lehmann & Zieger, 1977; Hehre et al., 1977, 1986; Kanda et al., 1986). Other evidence for the functional versatility of glycosylases comes from the finding that an inverting exo-α-glucanase (glucodextranase) catalyzes the hydration of 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol (D-*gluco*-heptenitol) to form 1-deoxy-β-D-*gluco*-heptulopyranose (β-D-heptulose), while promoting reactions leading to α-D-heptulosyl transfer products (Hehre et al., 1980; Schlesselmann et al., 1982). Finally, a clear example of protonation by two diferent catalytic groups of β-galactosidase was obtained with the aid of a new type of enolic glycosyl donor, (*Z*)-3,7-anhydro-1,2-dideoxy-D-*galacto*-oct-2-enitol. Lehmann and Schlesselmann (1983) found β-galactosidase to promote hydration of this octenitol by protonating it from the top (*re*) face, whereas the same enzyme had been shown to protonate D-galactal from below its *si* face (Lehmann & Zieger, 1977).

In view of the significant information obtained about β-galactosidase through its reaction with D-*galacto*-octenitol, the D-*gluco* analogue was prepared (Brewer et al., 1984) in the

† This study was supported by U.S. Public Health Service Research Grant GM-25478 (to E.J.H.) from the National Institute of General Medical Sciences and by Deutsche Forschungsgemeinschaft (to J.L.). Support for the NMR Facility at the Albert Einstein College of Medicine was provided by Instrumentation Grant I-S10-RR02309 from the National Institutes of Health and Grant DMB-8413123 from the National Science Foundation.
* Author to whom correspondence should be addressed.
‡ Department of Microbiology and Immunology, Albert Einstein College of Medicine.
§ Research Associate in Microbiology and Immunology, Albert Einstein College of Medicine, on leave from the Faculty of Agriculture, Hokkaido University, Sapporo, Japan.
‖ Department of Molecular Pharmacology, Atran Foundation Laboratory, Albert Einstein College of Medicine. Supported by U.S. Public Health Service Research Grant CA-16054 and Core Grant P30-13330 from the National Cancer Institute, National Institutes of Health.

0006-2960/88/0427-2294$01.50/0 © 1988 American Chemical Society