Biochemistry

 Volume 29, Number 6 February 13, 1990

Accelerated Publications

Iron(II)–Ethylenediaminetetraacetic Acid Catalyzed Cleavage of RNA and DNA Oligonucleotides: Similar Reactivity toward Single- and Double-Stranded Forms

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Received November 6, 1989; Revised Manuscript Received December 7, 1989

ABSTRACT: Fe(II)–EDTA catalyzes the cleavage of nucleic acids with little or no base-sequence specificity. We have now studied the preference of this reagent in catalyzing the cleavage of single- versus double-stranded nucleic acid structures. Three RNA and two DNA molecules, each expected to contain both single- and double-stranded regions, were synthesized and their structures characterized by enzymatic digestion using secondary structure specific nucleases. Fe(II)–EDTA catalyzed nearly uniform strand scission along the entire length of each molecule; no correlation with secondary structure was observed. The homopolymer sequence dA_{30}:dT_{30}, embedded in a mixed-sequence context to promote exact register of the homopolymer tract, was cleaved to an extent similar to that of flanking sequences. The reactions were relatively insensitive to K^+, Na^+, and Mg^{2+} in the range 10–100 mM and were quenched by Tris-HCl buffer. We conclude that the Fe(II)–EDTA-catalyzed strand scission reaction does not discriminate between typical single- and double-stranded regions, which simplifies the interpretation of experiments in which the reaction is used to probe the tertiary structure of RNA molecules [Latham, J. A., & Cech, T. R. (1989) *Science 245*, 276–282].

Free-radical-induced strand scission of nucleic acids has acquired widespread use as a cleavage method. In contrast to other chemical- and enzyme-based reagents, free radicals cleave nucleic acids with little or no base-sequence specificity. The chemical basis for this promiscuous reactivity lies in the strand scission reaction being initiated at the sugar moieties of the polynucleotide chain. Other chemical- and enzyme-based cleavage reagents owe their nucleotide-base specificity to their initial reaction with or recognition of the base itself. The ability of free radicals to react indiscriminately with all accessible sugar moieties in a given nucleic acid is an attractive feature for many applications of a strand scission reagent.

The reductive chemistry of Fe(II)–EDTA metal coordination complexes has been employed routinely to generate free radicals. Fe(II)–EDTA tethered to methidium, distamycin, or oligonucleotides has been used to promote double-strand-specific and in some cases sequence-specific cleavage of DNA (Dervan, 1986; Hertzberg & Dervan, 1984; Chu & Orgel, 1985; Dreyer & Dervan, 1986; Moser & Dervan, 1987) and RNA (Vary & Vournakis, 1984; Kean et al., 1985; Tanner & Cech, 1985; Lin et al., 1989). In its nonderivatized form, the Fe(II)–EDTA complex has been used as a solvent-based reagent to provide detailed information about protein–nucleic acid interactions (Tullius & Dombroski, 1985, 1986) and unusual nucleic acid conformations (Burkhoff & Tullius, 1987).

Latham and Cech (1989) recently utilized the free Fe(II)–EDTA complex as a solvent-based reagent to study the solution structures of mature $tRNA^{Phe}$ and a catalytic form of the *Tetrahymena* group I intron. Each RNA structure displayed areas of protection as well as regions of uniform cleavage by the reagent. Cleaved positions were taken as indications of those regions of the RNA molecule that were accessible to the solvent-based, free-radical reagent. Consistent with the expected behavior of a solvent-based reagent, strand scission catalyzed by the Fe(II)–EDTA complex occurred independent of the known secondary structure of the RNA molecules.

The notion that the Fe(II)–EDTA complex functions as a solvent-based reagent has been questioned by Jezewska et al. (1989). They reported that Fe(II)–EDTA-catalyzed strand

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0006-2960/90/0429-1355$02.50/0

scission occurred more rapidly on the annealed, duplex structure $dA(pA)_{69}$:$dpT(pT)_{69}$ than with either DNA strand in isolation. This result is not expected for a diffusible hydroxyl radical or other free-radical-reactive species in light of the unlikelihood that the sugar moieties of single- and double-stranded nucleic acids would differ dramatically in their accessibility to solvent. On the basis of their observation that KCl inhibited the cleavage of the duplex structure, Jezewska et al. (1989) suggest that Fe(II) may interact preferentially with the duplex structure prior to generation of the reactive species. These authors argue that Fe(II) affinity, rather than solvent accessibility, accounts for the observed strand cleavage of nucleic acids by Fe(II)–EDTA.

We have now analyzed the Fe(II)–EDTA cleavage of simpler RNA molecules than used by Latham and Cech (1989). Three RNA molecules, each containing one defined duplex structure and at least one defined single-stranded region, were utilized. We observe no significant secondary structure preference in radical-induced strand scission of the RNA structures. Extension of this analysis to two DNA molecules that adopt partially duplex structures also revealed no secondary structure dependence in the Fe(II)–EDTA-catalyzed strand breakage.

Materials and Methods

Description and Preparation of Nucleic Acids. The oligoribonucleotides were prepared with T_7 RNA polymerase directed transcription (Milligan et al., 1987). HIV TAR, transcribed from the plasmid pT_7HIVTAR (D. W. Celander, unpublished results), is a 103 nucleotide long synthetic transcript containing a portion (nucleotides −4 to +81) of the human immunodeficiency virus type 1 TAT-responsive element RNA (Rosen et al., 1985; Muesing et al., 1987). The other RNA oligonucleotides used in this study were provided by Drs. J. Gott (JM12) and M. Fedor and H. Heus (HH-S5). These oligonucleotide sequences are illustrated in Figure 1a–c; in each case, the RNA is folded into the secondary structure that is thought to be biologically active (Carey et al., 1983; Ruffner et al., 1989; Muesing et al., 1987).

The deoxyribonucleic acid molecules were prepared by phosphoramidite chemistry on an Applied Biosystems 380B DNA synthesizer. The synthetic DNA oligonucleotides used in this study are illustrated in Figure 1d,e.

All oligonucleotides were 5′-^{32}P-labeled with T_4 polynucleotide kinase and [γ-^{32}P]ATP as described (Maniatis et al., 1982). The oligoribonucleotides were dephosphorylated with calf intestinal alkaline phosphatase prior to kinase treatment. All 5′-^{32}P-labeled oligonucleotides were purified by fractionation on 12% or 20% polyacrylamide–50% (w/v) urea gels. The band corresponding to the full-length product was excised from the gel and crushed, and the nucleic acid was eluted from the gel material into 3 mL of 0.25 M NaCl–0.01 M Tris-HCl (pH 7.5)–0.001 M Na_2EDTA. The gel material was removed from the eluted nucleic acid on a 5-mL spin column, and the nucleic acid was precipitated with 3 volumes of ethanol. The nucleic acid was collected by centrifugation, dried in vacuo, and resuspended in 0.010 M Tris-HCl (pH 7.5)–0.001 M Na_2EDTA.

Sequencing Standards. Oligonucleotide sequencing ladders served as marker standards in the structure-mapping experiments. For the RNA oligonucleotides, sequencing ladders were generated by limited hydrolysis with RNases T_1 and U_2 and alkali according to the method of Donis-Keller et al. (1977).

For the DNA oligonucleotides, cleavage reactions specific for adenine + guanine, guanine, and thymine were done with

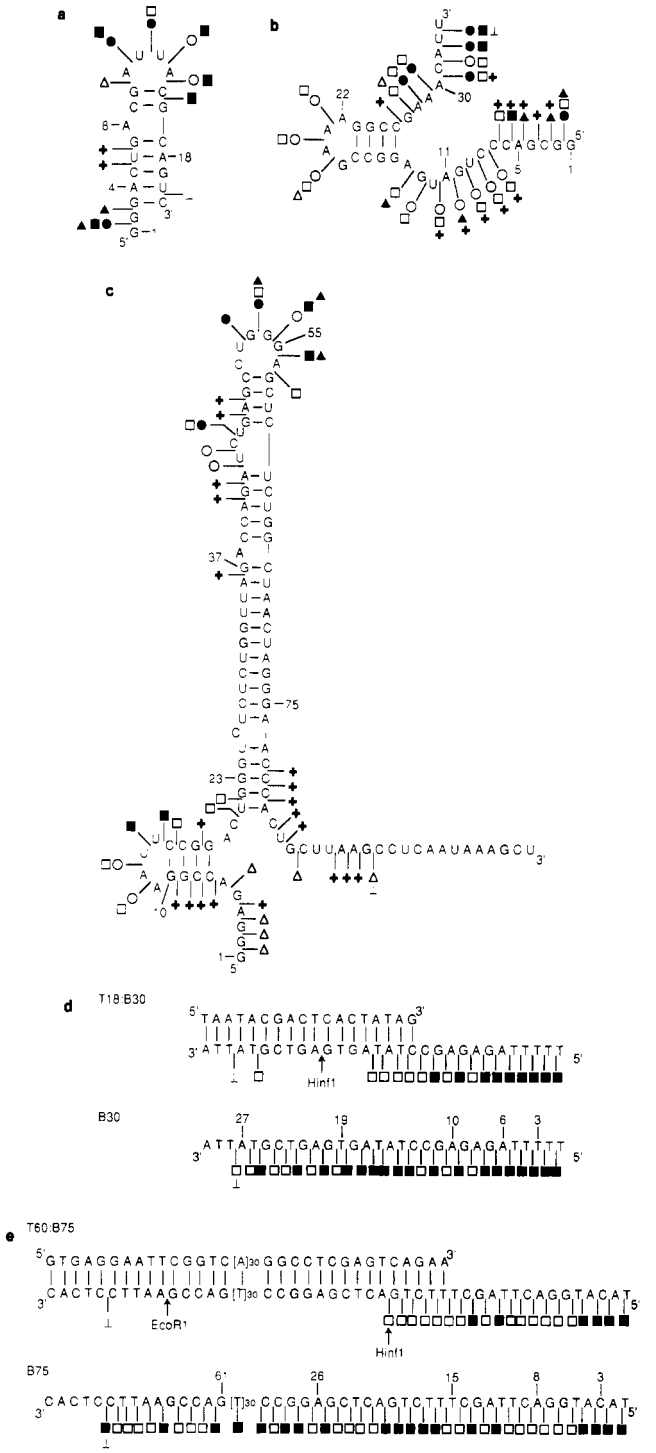

FIGURE 1: Structure of oligonucleotides used in this study. RNA oligonucleotides JM12 (a), HH-S5 (b), and HIV TAR (c) and DNA oligonucleotides B30 and T18:B30 (d) and B75 and T60:B75 (e) are illustrated. Secondary structure models are taken or inferred from the following sources: (a) Cary et al. (1983); (b) Ruffner et al. (1989); (c) Muesing et al. (1987); (d) Milligan et al. (1987). Indicated are the positions of weak (Δ, ○, □) and strong (▲, ●, ■, +) phosphodiester bond cleavage by single-strand-specific nucleases T_1 (▲), T_2 (●), and P_1 (■) and double-strand-specific RNase V_1 (+). The sites of cleavage by the restriction endonucleases Hin*f*1 and *Eco*RI are indicated by the arrows. The 3′-most nucleotide analyzed from the secondary structy mapping experiments for each 5′-^{32}P-labeled molecule is denoted by the symbol (⊥).

slight modification of the standard procedures (Maxam & Gilbert, 1980; Rubin & Schmid, 1980). In the modified procedure, strand scission was accomplished immediately following the base modification reaction. To each sample, pyrrolidine was added to a final concentration of 1 M, and

the sample was incubated at 90 °C for 15 min (Shi & Tyler, 1989). Following the heat treatment, each sample was lyophilized to dryness, and the nucleic acid was resuspended in 25 μL of water and lyophilized to dryness twice more.

Annealing Conditions and Structure Mapping of Nucleic Acids. The oligonucleotides were renatured for 0.5–10 h at 0–4 °C in 50 μL of annealing solution containing 0.050 M Tris-HCl (pH 7.5)–0.010 M $MgCl_2$–0.050 M NaCl following brief heat denaturation (95 °C, 2 min). The final concentration of nucleic acid in the annealing solution was estimated to be 25 nM.

The secondary structures of the RNA oligonucleotides were characterized with the single-strand-specific nucleases T_1, T_2, and P_1 as well as the double-strand-specific cobra venom nuclease V_1. The secondary structures of the DNA oligonucleotides were characterized with single-strand-specific nuclease P_1 and double-strand-specific type II restriction endonucleases. All structure-mapping experiments were done in a final 10-μL volume containing 7 μL of the oligonucleotide in annealing solution and 3 μL of enzyme solution. To confirm the presence of weakly and strongly cleaved sites within each oligonucleotide as well as to establish conditions of partial enzymatic digestion for each oligonucleotide, the nuclease concentration was varied over a 3–1000-fold range, and incubation times were performed over 2–60 min. All mapping reactions were terminated by addition of an equal volume of 2× loading buffer [0.020 M Na_2EDTA (pH 8.0)–10 M urea in 0.50× TBE] followed by freezing of the mixtures at −70 °C. The products were analyzed on 12% or 20% acrylamide–50% (w/v) urea gels.

Fe(II)–EDTA-Catalyzed Strand Scission of the Nucleic Acids. Following renaturation of a particular oligonucleotide in annealing buffer, a portion (7 μL) was mixed with 1 μL of 0.010 M $(NH_4)_2Fe(SO_4)_2$–0.020 M Na_2EDTA (pH 8.0), 1 μL of 0.010 M sodium ascorbate, and 1 μL of 0.6% H_2O_2. The cutting reactions were incubated at 20 °C for 10 min followed by the addition of 1 μL of 0.100 M thiourea, which serves to quench the free-radical reaction (Burkhoff & Tullius, 1987). Similar cleavage patterns were obtained when the cutting reactions were performed on the oligonucleotides for different incubation periods (2, 5, or 10 min) and under a variety of conditions, which included variations in Fe(II)–EDTA, sodium ascorbate, and H_2O_2 concentrations (not shown). An equal volume of 2× loading buffer was added to each sample, and electrophoresis was performed on a 12% or 20% acrylamide–50% (w/v) urea gel.

To assess the effect of the buffer components Tris–HCl, NaCl, KCl, and $MgCl_2$ on the cleavage of oligonucleotides by the Fe(II)–EDTA reagent, the annealing and subsequent cleavage reactions were performed in the following fashion. Five microliters of the HH-S5 RNA oligonucleotide [suspended in 0.01 M Tris-Cl (pH 7.5)–0.001 M Na_2EDTA] was heat denatured (90 °C, 2 min) and renatured through the addition of 2 μL of a 5-fold concentrated stock solution of the desired renaturation buffer. The cleavage reaction for the renatured oligonucleotide was initiated by sequential addition of 1 μL each of the following components: 0.001 M $(NH_4)_2Fe(SO_4)_2$–0.002 M Na_2EDTA (pH 8.0), 0.010 M sodium ascorbate, and 0.6% H_2O_2. The cutting reaction was performed at 20 °C for 10 min, followed by addition of 1 μL of 0.100 M thiourea.

Results

RNA. We assessed the secondary structure specificity of the Fe(II)–EDTA cutting reagent using RNA molecules that were derived from biological sources and are thought to possess defined biological or biochemical function in their folded structure. The JM12 RNA is a variant of the bacteriophage R17 replicase mRNA leader sequence that is bound by the bacteriophage coat protein (Romaniuk et al., 1987). The HH-S5 RNA is a 3/4 catalytic RNA subunit that is capable of cleaving substrate RNA molecules in a sequence-specific fashion (Ruffner et al., 1989). The HIV TAR RNA contains a portion of the human immunodeficiency virus type 1 TAT-responsive element (Rosen et al., 1985; Muesing et al., 1987). Each of these RNA molecules forms a defined secondary structure, consisting of regions of single- and double-strand character (Carey et al., 1983; Muesing et al., 1987; Ruffner et al., 1989); the anticipated structure of each RNA molecule is illustrated in Figure 1a–c.

Each molecule's secondary structure was characterized with various single- and double-strand-specific nucleases. The results of the nuclease studies on the JM12, HH-S5, and HIV TAR RNA molecules are illustrated in panels a–c of Figure 2 and summarized in panels a–c of Figure 1. Three types of structural regions were found in these RNA molecules, as follows.

(*1*) *Single-Stranded Regions.* The digestion patterns displayed by the single-strand-specific T_2 and P_1 nucleases coincided for each RNA examined. Those nonpaired guanosines that were moderately cleaved by T_2 and P_1 nucleases were also cleaved by the T_1 nuclease. Despite differences in base specificity and chemical mechanism of cleavage, these three nucleases recognize similar regions. Thus, sequences shown as single stranded in Figure 1 that were cleaved by these nucleases and not by the double-strand-specific V_1 nuclease were assigned as single stranded.

(*2*) *Double-Stranded Regions.* Double-stranded RNA content is clearly indicated if a region is readily digested by V_1 nuclease yet remains intact upon incubation with single-strand-specific nucleases. In only limited regions of the three RNA molecules studied did this differential sensitivity toward enzymatic digestion confirm the presence of the double-stranded RNA. Inefficient V_1 nuclease digestion in regions of putative double-strand character was routinely observed, consistent with the reported properties of the nuclease (Lockard & Kumar, 1981; Favorova et al., 1981). Thus, the assignment of double-stranded regions relied more heavily on the previous analysis of the biologically active structure and on the absence of cleavage by single-strand-specific nucleases.

(*3*) *Ambiguous Regions.* The V_1 nuclease cleaved the RNA molecules in some regions illustrated in Figure 1b,c to be single stranded [(panel b) residue positions 2–11 and 30; (panel c) residue positions 4, 6, 82, 83, and 87–89]; consequently, we are uncertain about whether these regions are single stranded as depicted in Figure 1 or whether these regions are actually double stranded with unconfirmed pairing partners. This ambiguity may be attributed to the ability of V_1 nuclease to cleave the phosphate backbone of a stacked base independent of the base's involvement in a Watson–Crick pairing (Lockard & Kumar, 1981; Auron et al., 1982; Lowman & Draper, 1986; Puglisi et al., 1988). The nuclease digestion data summarized in Figure 1 (a and c) are consistent with previous structure-mapping experiments performed on the RNA molecules (Carey et al., 1983; Muesing et al., 1987).

To determine whether the Fe(II)–EDTA reagent displayed any secondary structure preference in cleaving RNA, we performed the free-radical cutting reaction on the RNA molecules under the same conditions employed for the structure-mapping experiments. In contrast to the structure-specific nucleases, Fe(II)–EDTA catalyzed nearly uniform strand

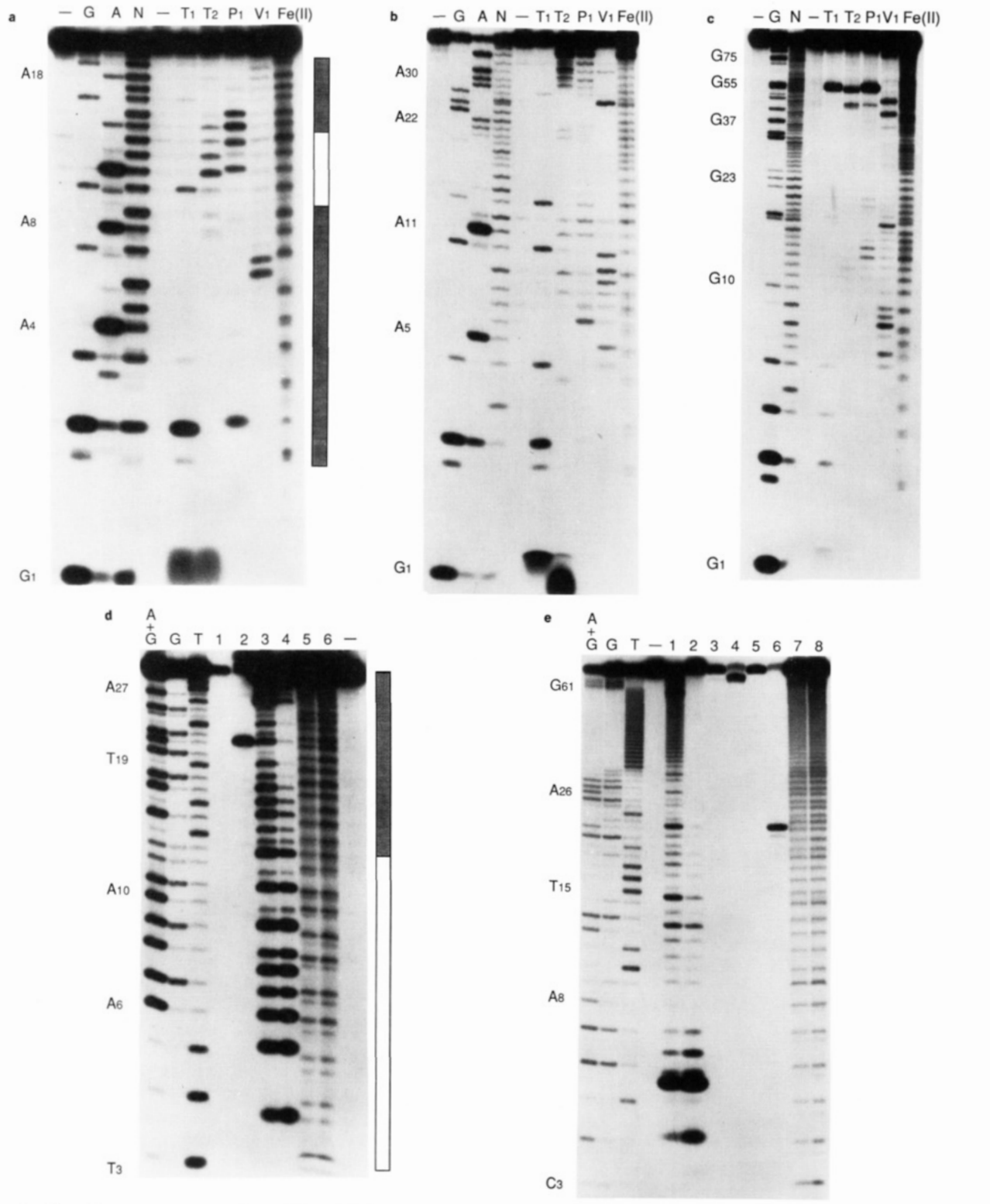

FIGURE 2: Secondary structure mapping and iron(II)–EDTA cleavage of RNA and DNA oligonucleotides. The results of nuclease and iron(II)–EDTA cleavage of JM12 RNA (a), HH-S5 RNA (b), and HIV TAR RNA (c) are shown. The lanes denoted by G, A, and N represent sequencing ladders specific for guanosine, adenosine, or any nucleotide, respectively. The lanes containing RNA samples incubated under native conditions with either no added cleavage reagent (–), ribonuclease T_1 (T_1), ribonuclease T_2 (T_2), nuclease P_1 (P_1), ribonuclease V_1 (V_1), or the Fe(II)–EDTA reagent [Fe(II)] are indicated. The results of the nuclease and Fe(II)–EDTA cleavage of B30 DNA and T18:B30 DNA (d) and of B75 DNA and T60:B75 DNA (e) are indicated. The lanes denoted by A+G, G, and T represent sequencing ladders specific for the indicated bases. In (d), lanes containing DNA samples incubated with either no added cleavage reagent (–), *Hinf*I (1 and 2), nuclease P_1 (3 and 4), or the Fe(II)–EDTA reagent (5 and 6) are shown. In (e), lanes containing DNA samples incubated with either no added cleavage reagent (–), nuclease P_1 (1 and 2), *Eco*RI (3 and 4), *Hinf*I (5 and 6), or the Fe(II)–EDTA reagent (7 and 8) are shown. In (d) and (e), the odd-numbered lanes indicate the samples containing the single-stranded molecules, and the even-numbered lanes indicate the samples containing the heteroduplex molecules. The vertical bar shown adjacent to the Fe(II)–EDTA cleavage lane in (a) and (d) illustrates the location of the single-stranded (open) and double-stranded (shaded) regions relative to the products of Fe(II)–EDTA cleavage. Note that in RNA [exemplified here in (a)], the assignment of product identity in Fe(II)–EDTA cleavage is shifted one nucleotide 5′ of the actual site of cleavage relative to the alkaline hydrolysis product since only strand scission by Fe(II)–EDTA is accompanied by elimination of the ribose moiety. In DNA [exemplified here in (d)], the assignment of product identity in Fe(II)–EDTA cleavage corresponds directly to the base identity in the sequencing ladders since strand cleavage by both the Fe(II)–EDTA reagent and the chemical sequencing methodologies results in elimination of the deoxyribose moiety.

Table I: Distribution of Cleavage Products in Single- and Double-Stranded Regions[a]

oligonucleotide	catalyst	backbone positions quantitated	relative distribution of fragmentation products		
			overall[b]	single stranded[c]	double stranded[c]
JM12 RNA	hot alkali	2–18	100 ± 47 (46, 211)	*d*	*d*
	Fe(II)–EDTA	3–19	100 ± 41 (45, 207)	123 ± 57 (77, 207)	93 ± 34 (45, 149)
HH-S5 RNA	hot alkali	2–33	100 ● 61 (29, 268)	*d*	*d*
	Fe(II)–EDTA	3–30	100 ± 38 (56, 209)	115 ± 42 (62, 185)	82 ± 20 (68, 182)
HIV TAR RNA	hot alkali	2–71	100 ± 69 (10, 300)	*d*	*d*
	Fe(II)–EDTA	3–75	100 ± 51 (26, 271)	101 ± 46 (26, 233)	101 ± 54 (32, 271)
B30 DNA	Fe(II)–EDTA	3–27	100 ± 32 (48, 170)	101 ± 39 (48, 170)[e]	99 ± 27 (65, 160)[e]
T18:B30 DNA	Fe(II)–EDTA	3–28	100 ± 27 (39, 151)	84 ± 30 (39, 122)	110 ± 20 (78, 151)
B75 DNA	Fe(II)–EDTA	3–56	100 ± 47 (31, 222)	108 ± 34 (47, 174)[e]	99 ± 50 (31, 222)[e]
T60:B75 DNA	Fe(II)–EDTA	3–56	100 ± 46 (43, 207)	116 ± 39 (68, 187)	97 ± 46 (43, 207)

[a] Quantitation was achieved by scanning of autoradiographic films with an LKB Ultroscan XL enhanced laser densitometer and integration with an LKB gel scan XL laser densitometer software program. [b] Normalized mean distribution ● standard deviation of cleavage extent among fragmentation products integrated. The low and high values that delimit the range of the distribution are given in parentheses. [c] The values reported are relative to the normalized, overall extent of fragmentation. A region was denoted as single stranded or double stranded on the basis of the molecule structures depicted in Figure 1. Regions of uncertain structure (HH-S5 RNA, backbone positions 3–11 and 30; HIV TAR RNA, backbone positions 4 and 6), which were integrated and represented in the overall distribution of fragmentation products, were excluded in the analysis of the relative distribution of fragmentation products that are single stranded. [d] Not determined. [e] To facilitate comparison of selected regions of B30 with T18:B30 and of B75 with T60:B75, the column denoted as single stranded refers to those regions that are single stranded in the heteroduplexes, while the column denoted as double stranded refers to those regions that are double stranded in the heteroduplexes.

scission along the entire length of each tested molecule (Figure 2a–c). Quantitative analysis of the strand scission products indicated that in most cases the distribution among ribose fragmentation products varied by less than 60% (Table I). Similar nucleic acid cleavage patterns were obtained with the Fe(II)–EDTA reagent when the cleavage assay was performed under a variety of reaction conditions, which included variations in Fe(II)–EDTA and H_2O_2 concentrations and reaction time (data not shown). The pattern of variation of cleavage produced by the Fe(II)–EDTA reagent is similar to that generated by hot alkali (Figure 2a–c; Table I).

DNA. To determine whether the lack of secondary structure preference in strand scission by the Fe(II)–EDTA reagent was limited to RNA or a general property of nucleic acids, we evaluated the reagent's ability to catalyze cleavage on structurally defined DNA molecules. We tested two different types of DNA molecules. The molecule designated as T18:B30 is an annealed heteroduplex composed of an 18 base pair, double-stranded region and a 12 nucleotide long single-stranded region (Figure 1d); the double-stranded region of this molecule composes the promoter element for accurate and efficient transcription by T_7 RNA polymerase. The second molecule examined, denoted as T60:B75, is an annealed heteroduplex molecule composed of a 60 base pair, double-stranded region and a 15 nucleotide long single-stranded region (Figure 1e). Within the duplex region of T60:B75, a tract of 30 A-T base pairs is flanked by two 15 base pair tracts of mixed sequence composition (Figure 1e).

As with characterization of the RNA molecules, structure-specific enzyme reagents were used to analyze the secondary structure of these DNA molecules. The structure of the DNA molecule denoted as T18:B30 was characterized with a type II restriction endonuclease and P_1 nuclease. The sequence of B30 includes a *Hinf*1 restriction enzyme cleavage site. Since the *Hinf*1 restriction endonuclease cleaves its recognition sequence more rapidly on duplex DNA relative to single-stranded DNA, we employed this enzyme as a sensitive probe of double-stranded character at least for the region of the T18:B30 molecule that contains its cognate recognition site. As shown in Figure 2d, the annealed T18:B30 heteroduplex is cleaved efficiently and accurately by the enzyme while the single-stranded B30 molecule remained uncut under the same conditions (Figure 2d, lanes 1 and 2). Nuclease P_1 was used to provide complementary information about the single-stranded content of the B30 and T18:B30 molecules. The B30 molecule was digested nearly uniformly by P_1 nuclease while the T18:B30 heteroduplex molecule was digested preferentially in the 5′-most 12–16 nucleotides of the labeled B30 strand (Figure 2d, lanes 3 and 4). These nuclease digestion patterns are consistent with the structure of T18:B30 being represented as an annealed heteroduplex containing a single-stranded 5′-tail (Figure 1d).

The structure of the DNA molecule designated as T60:B75 was similarly characterized with type II restriction endonucleases and nuclease P_1. The T60:B75 duplex molecule was cleaved accurately and efficiently by *Eco*RI and *Hinf*1 endonucleases (Figure 2e, lanes 4 and 6). Under identical incubation conditions, each endonuclease failed to cleave the B75 single-stranded molecule (Figure 2d, lanes 3 and 5). Conversely, a nearly uniform DNA cleavage pattern was generated when the B75 single-stranded molecule was incubated with nuclease P_1, whereas only the 5′-most 15–20 nucleotides of the T60:B75 duplex molecule were susceptible to digestion by the nuclease under identical conditions (Figure 2d, lanes 1 and 2). The results of structure-mapping experiments support the structure of B75 being represented as a single-stranded molecule and the structure of T60:B75 being represented as the heteroduplex molecule, as illustrated in Figure 1e.

Fe(II)–EDTA-catalyzed of these DNA molecules was performed under the same conditions employed for the structure-mapping experiments. As shown in Figure 2d for the first DNA molecule, there was no preferential cleavage of the B30 strand in the duplex region relative to the single-stranded tail of T18:B30 (lane 6). Furthermore, no significant increase in reactivity toward the B30 strand was seen when present in its partially annealed form as the heteroduplex T18:B30 molecule as compared with its fully single-stranded form (Figure 2d, compare lane 6 with lane 5). Similar cleavage results were obtained with the B75 single-stranded molecule and the T60:B75 heteroduplex molecule (Figure 2e, lanes 7 and 8). Quantitative analysis of the cleavage product distributions confirmed that the Fe(II)–EDTA-catalyzed strand scission varied by less than 50% among the deoxyribose moieties in most cases (Table I).

Salt and Buffer Dependence of Cleavage. The effect of various buffer components on the Fe(II)–EDTA-catalyzed cutting reaction of the HH-S5 RNA oligonucleotide was determined. The monovalent salts NaCl and KCl and the di-

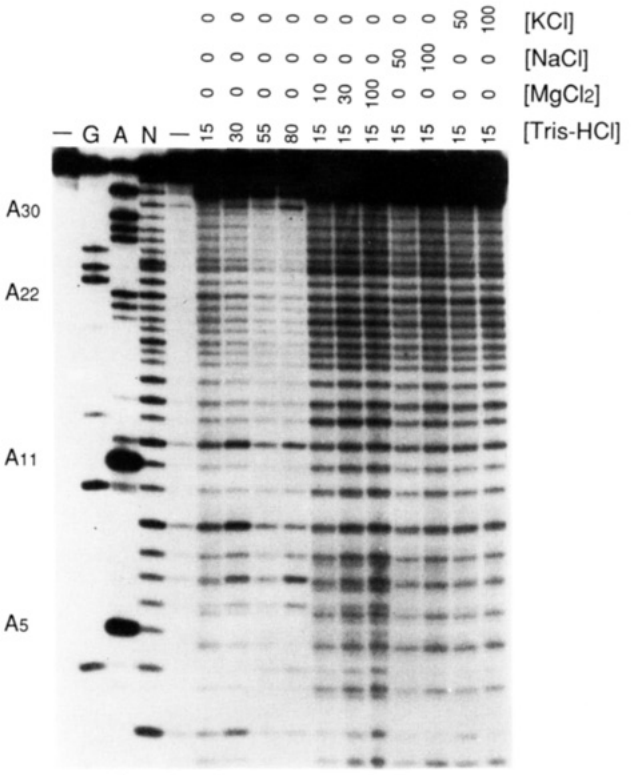

FIGURE 3: Effect of buffer components on iron(II)–EDTA cleavage of the HH-S5 RNA oligonucleotide. The sequencing lanes are denoted as in Figure 2. The lane containing the oligonucleotide sample incubated with no added cleavage reagent is denoted by (–). The other lanes indicate the final buffer component concentrations (each expressed in mM) in which the oligonucleotide was subjected to the Fe(II)–EDTA-catalyzed cleavage reaction.

valent salt $MgCl_2$ had no effect upon the cleavage reaction on the oligonucleotide at salt concentrations as high as 100 mM (Figure 3). The buffering component, Tris-HCl, quenched the cutting reaction on the oligonucleotide at concentrations beginning at 30 mM (Figure 3). Similar results concerning the effect of these solution components upon the Fe(II)–EDTA-catalyzed cleavage of a partially duplex DNA oligonucleotide were also obtained [data not shown; see also Tullius et al. (1987)].

Discussion

We have investigated whether the Fe(II)–EDTA reagent displays any secondary structure preference in catalyzing RNA strand scission by free radicals. Each of the three RNA molecules analyzed contained clearly evident single-stranded and double-stranded regions. Although there was modest variation of the extent of cleavage along each chain, no significant correlation of cleavage with single or double strandedness was observed (Table I). The variation of Fe(II)–EDTA-catalyzed cleavage was similar to that produced by alkali-catalyzed cleavage, suggesting that neighboring base sequence has a modest effect on strand cleavage by both reagents.

The Fe(II)–EDTA reagent catalyzes RNA strand cleavage as expected for a solvent-based reagent. The cleavage data on RNA molecules composed of only simple secondary structures provide a foundation upon which a description of the reagent's cleavage activity on more complex RNA molecules may be interpreted. As inferred previously by Latham and Cech (1989), the lack of reaction of certain regions of more complex RNA molecules with this reagent must reflect protection afforded by RNA tertiary structure. Tertiary structure can make portions of the phosphodiester backbone inaccessible to the reagent or perhaps even inaccessible to solvent.

One of the molecules studied here, HH-S5 RNA, is a catalytic oligoribonucleotide of the type described by Haseloff and Gerlach (1988). In spite of the likelihood that a defined tertiary structure is required for catalytic activity of this small oligoribonucleotide and in contrast to that observed with a catalytic form of the *Tetrahymena* group I intron (Latham & Cech, 1989), no backbone protection of HH-S5 RNA is seen with the Fe(II)–EDTA reagent. It remains to be seen whether backbone protection of HH-S5 RNA would appear upon incubation of this catalytic oligoribonucleotide with its substrate strand.

As observed with RNA, Fe(II)–EDTA catalyzed nearly uniform strand breakage in single- and double-stranded regions of DNA. We consider the oligodeoxyribonucleotides used in this study to be valid examples of the types of mixed nucleotide sequence composition expected to occur naturally. An important conclusion from our results is that the apparent cleavage specificity of Fe(II)–EDTA for the duplex DNA inferred from experiments with $dA(pA)_{69}$:$dpT(pT)_{69}$ by Jezewska et al. (1989) is not a general feature of the reagent for duplex DNA molecules. Furthermore, we did not observe such secondary structure specificity in the reagent's action on a DNA molecule that contained a tract of 30 A-T base pairs (Figure 2e, lanes 7 and 8). We conclude that secondary structure specificity of the Fe(II)–EDTA reagent is not even an intrinsic property of duplex A-T DNA. We consider that the results obtained by Jezewska et al. (1989) may be better attributed to some unusual feature of the nucleic acid they studied than to some mechanistic aspect of the cleavage reagent.

The similar reactivity toward single- and double-stranded forms of RNA and DNA observed with Fe(II)–EDTA distinguishes this reagent from Cu(I)–1,10-phenanthroline. The latter complex causes strand scission preferentially in duplex, B-form DNA (Marshall et al., 1981; Pope & Sigman, 1984; Drew, 1984) but induces cleavage predominantly in single-stranded regions of RNA (Murakawa et al., 1989).

The Fe(II)–EDTA reagent catalyzes strand scission of RNA and DNA similarly, and this behavior is also documented in the reagent's activity in different buffer systems. The buffer component, Tris, presumably serving as a free-radical scavenger, quenched the strand scission reaction. Cleavage occurred to similar extents in Na^+, K^+, and Mg^{2+} at concentrations as high as 100 mM. Although we observe no effect of moderate $MgCl_2$ concentrations upon the strand scission of RNA molecules of simple structure, the pattern of cleavage on other RNAs may be affected greatly by Mg(II). In the case of Fe(II)–EDTA-catalyzed cleavage of $tRNA^{Phe}$ (Latham & Cech, 1989), the protection pattern observed in certain regions of the molecule may be attributed to occupancy of a specific magnesium coordination site(s) in the stable tertiary structure. Divalent cations coordinate to $tRNA^{Phe}$ at specific metal binding sites, which results in stabilization of the molecule's tertiary structure (Danchin, 1972; Kayne & Cohn, 1972; Jack et al., 1977; Holbrook et al., 1977; Quigley et al., 1978). One exciting prospect will be to determine whether specific metal binding sites can be identified in RNA tertiary structures by the Fe(II)-EDTA reagent in conjunction with other methodologies.

Acknowledgments

We gratefully acknowledge Marty Fedor, Jonatha Gott, and Hans Heus for oligoribonucleotides, Art Zaug for generous

gifts of T_7 RNA polymerase, Jenny Nyborg for a plasmid that contained an HIV type 1 long terminal repeat element, and Cheryl Grosshans for oligodeoxyribonucleotides. We thank Rob Kuchta for advice and use of the densitometer and associated software.

References

Auron, P. E., Weber, L. D., & Rich, A. (1982) *Biochemistry 21*, 4700–4706.
Burkhoff, A. M., & Tullius, T. D. (1987) *Cell 48*, 935–943.
Carey, J., Cameron, V., de Haseth, P. L., & Uhlenbeck, O. C. (1983) *Biochemistry 22*, 2601–2610.
Chu, B. C. F., & Orgel, L. E. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 963–967.
Danchin, A. (1972) *Biochimie 54*, 333–337.
Dervan, P. B. (1986) *Science 232*, 464–471.
Donis-Keller, H., Maxam, A. M., & Gilbert, W. (1977) *Nucleic Acids Res. 4*, 2527–2538.
Drew, H. R. (1984) *J. Mol. Biol. 176*, 535–557.
Dreyer, G. B., & Dervan, P. B. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 968–972.
Favorova, O. O., Fasiolo, F., Keith, G., Vassilenko, S. K., & Ebel, J. P. (1981) *Biochemistry 20*, 1006–1011.
Haseloff, J., & Gerlach, W. L. (1988) *Nature 334*, 585–591.
Hertzberg, R. P., & Dervan, P. B. (1984) *Biochemistry 23*, 3934–3945.
Holbrook, S. R., Sussman, J. L., Warrant, R. W., Church, G. M., & Kim, S. H. (1977) *Nucleic Acids Res. 4*, 2811–2820.
Jack, A., Ladner, J. E., Rhodes, D., Brown, R. S., & Klug, A. (1977) *J. Mol. Biol. 111*, 315–328.
Jezewska, M. J., Bujalowski, W., & Lohman, T. M. (1989) *Biochemistry 28*, 6161–6164.
Kayne, M., & Cohn, M. (1972) *Biochem. Biophys. Res. Commun. 46*, 1285–1291.
Kean, J. M., White, S. A., & Draper, D. E. (1985) *Biochemistry 24*, 5062–5070.
Latham, J. A., & Cech, T. R. (1989) *Science 245*, 276–282.
Lin, S. B., Blake, K. R., Miller, P. S., & Ts'o, P. O. P. (1989) *Biochemistry 28*, 1054–1061.
Lockard, R. E., & Kumar, A. (1981) *Nucleic Acids Res. 9*, 5125–5140.
Lowman, H. B., & Draper, D. E. (1986) *J. Biol. Chem. 261*, 5396–5403.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning—A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Marshall, L. E., Graham, D. R., Reich, K. A., & Sigman, D. S. (1981) *Biochemistry 20*, 244–250.
Maxam, A. M., & Gilbert, W. (1980) *Methods Enzymol. 65*, 499–560.
Milligan, J. F., Groebe, D. R., Witherell, G. W., & Uhlenbeck, O. C. (1987) *Nucleic Acids Res. 15*, 8783–8798.
Moser, H. E., & Dervan, P. B. (1987) *Science 238*, 645–650.
Muesing, M. A., Smith, D. H., & Capon, D. J. (1987) *Cell 48*, 691–701.
Murakawa, G. J., Chen, C. B., Kuwabara, M. D., Nierlich, D. P., & Sigman, D. S. (1989) *Nucleic Acids Res. 17*, 5361–5375.
Pope, L. E., & Sigman, D. S. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3–7.
Puglisi, J. D., Wyatt, J. R., & Tinoco, I., Jr. (1988) *Nature 331*, 283–286.
Quigley, G. J., Teeter, M. M., & Rich, A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 64–68.
Romaniuk, P. J., Lowary, P., Wu, H., Stormo, G., & Uhlenbeck, O. C. (1987) *Biochemistry 26*, 1563–1568.
Rosen, C., Sodroski, J., & Haseltine, W. (1985) *Cell 41*, 813–823.
Rubin, C. M., & Schmid, C. W. (1980) *Nucleic Acids Res. 8*, 4613–4619.
Ruffner, D. E., Dahm, S. C., & Uhlenbeck, O. C. (1989) *Gene 82*, 31–41.
Shi, Y., & Tyler, B. M. (1989) *Nucleic Acids Res. 17*, 3317.
Tanner, N. K., & Cech, T. R. (1985) *Nucleic Acids Res. 13*, 7759–7779.
Tullius, T. D., & Dombroski, B. A. (1985) *Science 230*, 679–681.
Tullius, T. D., & Dombroski, B. A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5469–5473.
Tullius, T. D., Dombroski, B. A., Churchill, M. E. A., & Kam, L. (1987) *Methods Enzymol. 155*, 537–558.
Vary, C. P. H., & Vournakis, J. N. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6978–6982.