Mapping RNA Regions in Eukaryotic Ribosomes That Are Accessible to Methidiumpropyl-EDTA·Fe(II) and EDTA·Fe(II)†

Hogyu Han,‡ Alanna Schepartz,‡,§ Maria Pellegrini,*,∥ and Peter B. Dervan*,‡

Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125, and Molecular Biology Program, University of Southern California, Los Angeles, California 90089

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ABSTRACT: Methidiumpropyl-EDTA·Fe(II) [MPE·Fe(II)] and EDTA·Fe(II) were used to investigate the structure of *Drosophila melanogaster* ribosomes. Cleavage reactions were performed on intact ribosomes in cell lysates *in vitro* and analyzed by primer extension with reverse transcriptase using oligodeoxynucleotide primers. Regions of 18S and 28S ribosomal RNAs (rRNAs) which are accessible to MPE·Fe(II) and EDTA·Fe(II) are located almost exclusively within expansion segments. The accessibility of these regions to cleavage indicates that they are likely exposed on the surface of eukaryotic ribosomes. These results provide information about the overall tertiary structure of rRNA in ribosomes.

Ribosomes, ubiquitous ribonucleoproteins, require specifically folded RNA tertiary structures to carry out the essential process of protein biosynthesis in the cell. The rRNAs[1] in ribosomes interact directly with messenger RNA, transfer RNA, factors, and various ribosome-directed antibiotics and are involved in codon–anticodon interaction, the peptidyltransferase region, and subunit association (Dahlberg, 1989; Shine & Dalgarno, 1974; Ofengand et al., 1986; Moazed & Noller, 1986, 1987, 1989a,b; Barta et al., 1984; Moazed et al., 1988; Noller et al., 1992). Low-resolution three-dimensional models of 16S rRNA in the *Escherichia coli* small ribosomal subunit have been derived from methods such as chemical and enzymatic probing, comparative sequence analysis, biophysical approaches, and site-specific mutagenesis (Stern et al., 1988a, 1989; Brimacombe et al., 1988). In contrast to prokaryotic (especially *E. coli*) ribosomes, far less is known about rRNA conformations of eukaryotic ribosomes.

MPE·Fe(II), which consists of EDTA·Fe(II) tethered to the intercalator methidium, has proven useful for studying interactions between DNA binding molecules and DNAs as well as structures of RNAs (Hertzberg & Dervan, 1982, 1984; Van Dyke et al., 1982; Dervan, 1986; Vary & Vournakis, 1984; Tanner & Cech, 1985; Kean et al., 1985; White & Draper, 1989). Upon addition of dithiothreitol or sodium ascorbate plus hydrogen peroxide, the EDTA·Fe(II) moiety cleaves DNA and RNA by oxidation of the backbone via a nonspecific diffusible oxidant, most likely hydroxyl radical. Analysis of the cleavage products on a high-resolution denaturing polyacrylamide gel allows nucleotide positions accessible to the cleaving moiety to be mapped to nucleotide resolution.

Here, we report the use of MPE·Fe(II) in conjunction with primer extension for studies of 18S and 28S rRNA structures in eukaryotic ribosomes. EDTA·Fe(II), a solvent-based untethered reagent, was used for comparison (Tullius & Dombroski, 1985, 1986; Latham & Cech, 1989; Celander & Cech, 1991; Huttenhofer & Noller, 1992). Intact ribosomes from *Drosophila melanogaster* were studied since *D. melanogaster* is a eukaryote which is well characterized biochemically and genetically. The results reveal that cleavage occurs in a limited number of accessible regions of 18S and 28S rRNAs. The 18S and 28S rRNA regions that are susceptible to cleavage by MPE·Fe(II) and EDTA·Fe(II) are located within regions defined previously as "expansion segments". Accessibility to the cleaving agents indicates that these expansion segment regions are probably exposed on the surface of the intact eukaryotic ribosomes. The presence of certain surface-exposed rRNA sequences in eukaryotic ribosomes capable of actively translating *in vitro* has implications for the spatial arrangement of rRNAs in ribosomes.

MATERIALS AND METHODS

Preparation of the Embryo Lysate. The embryo lysate was prepared essentially as described by Scott et al. (1979). Two grams of freshly collected *D. melanogaster* embryos (0–20 h) were dechorionated in 100 mL of a 1:1 mixture of 95% ethanol and chlorox (2% sodium hypochlorite) for 1 min on ice. Embryos were then washed thoroughly with ice-cold phosphate-buffered saline (50 mM KH_2PO_4, pH 7.5, 100 mM NaCl) and pelleted at 1000*g* for 4 min at 0 °C. The pellet was resuspended in 2 mL of 10 mM Hepes, pH 7.6, and homogenized by 10–20 strokes of a loose-fitting Dounce homogenizer on ice. The homogenate was centrifuged at 40000*g* for 20 min at 0 °C. After centrifugation, the clear supernatant was removed and immediately used for reactions with chemical reagents.

MPE·Fe(II) and EDTA·Fe(II) Reactions with Ribosomes in Lysates. A reaction mixture containing 100 μL of lysate, 15 μL of the appropriate 10× concentration of preformed MPE·Fe(II) or EDTA·Fe(II), and 25 μL of H_2O was preincubated for 5 min at 25 °C. The cleavage reaction was initiated by addition of 5 μL each of freshly prepared 30× sodium ascorbate (30 mM) and 30× H_2O_2 (9%) solutions. The reaction was incubated for 30 min at 25 °C and stopped by addition of 850 μL of denaturing buffer (4 M guanidinium

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* To whom correspondence should be addressed.

‡ California Institute of Technology.

§ Present address: Department of Chemistry, Yale University, New Haven, CT 06511.

∥ University of Southern California.

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[1] Abbreviations: MPE, methidiumpropyl-EDTA; EDTA, ethylenediaminetetraacetic acid; rRNA, ribosomal RNA; Tris, tris(hydroxymethyl)aminomethane; nt, nucleotide.

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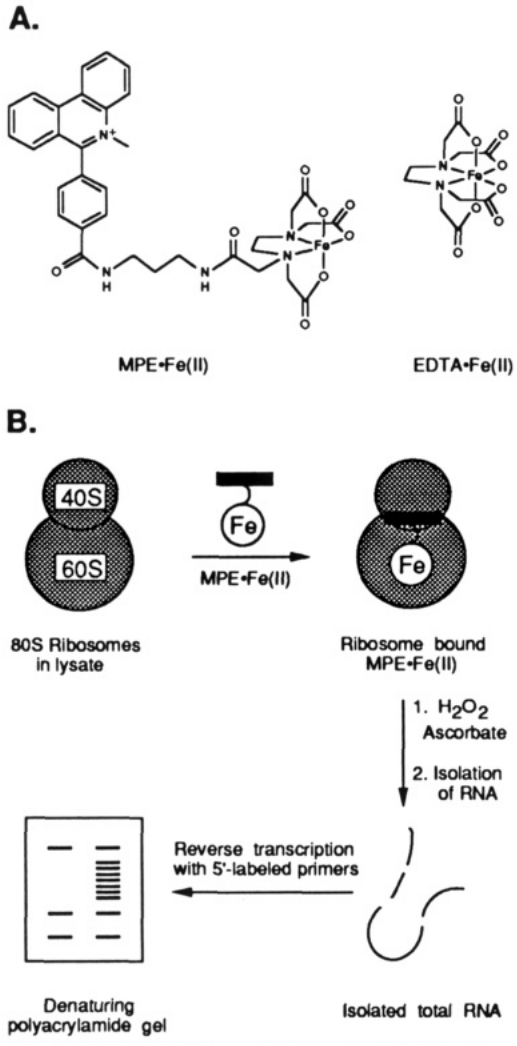


FIGURE 1: (A) MPE·Fe(II) and EDTA·Fe(II). (B) Scheme for cleavage of ribosomes by MPE·Fe(II).

thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl). The RNA was purified according to the procedure of Chomczynski and Sacchi (1987). The reaction mixture was transferred to a 15-mL polypropylene tube and kept on ice at all times unless otherwise indicated. Sequentially, 0.1 mL of 2 M sodium acetate, pH 4.0, 1 mL of water-saturated phenol, and 0.2 mL of a chloroform–isoamyl alcohol mixture (49:1) were added to the tube. The final suspension was mixed thoroughly, cooled on ice for 15 min, and centrifuged at 10000*g* for 20 min at 4 °C. After centrifugation, the aqueous phase was transferred to a prechilled tube and mixed with 2.5 volumes of cold 95% ethanol plus $^1/_3$ volume of 10 M NH_4OAc. The RNA was precipitated for 2 h at −70 °C and pelleted by centrifugation at 10000*g* for 20 min at 4 °C. The RNA pellet was dissolved in 0.3 mL of denaturing buffer and transferred to a 1.5-mL Eppendorf tube. The RNA was precipitated by addition of 900 μL of 95% cold ethanol and 100 μL of 10 M NH_4OAc. After centrifugation at 16000*g* for 30 min at 4 °C, the RNA pellet was washed with 1 mL of 70% ethanol, dried in a Savant Speed Vac concentrator, and resuspended at 1 μg/μL in water.

Primer Extension. Primer extension was performed as described (Moazed et al., 1986; Stern et al., 1988b). The DNA oligonucleotides were prepared by solid-phase synthesis on an Applied Biosystems Model 380B DNA synthesizer using β-cyanoethyl phosphoramidite chemistry. The oligonucleotides were deprotected by treatment with concentrated NH_4OH at 55 °C for 24 h and purified by gel electrophoresis on a denaturing 20% polyacrylamide gel. The oligonucleotides

A. Primers for 18S rRNA

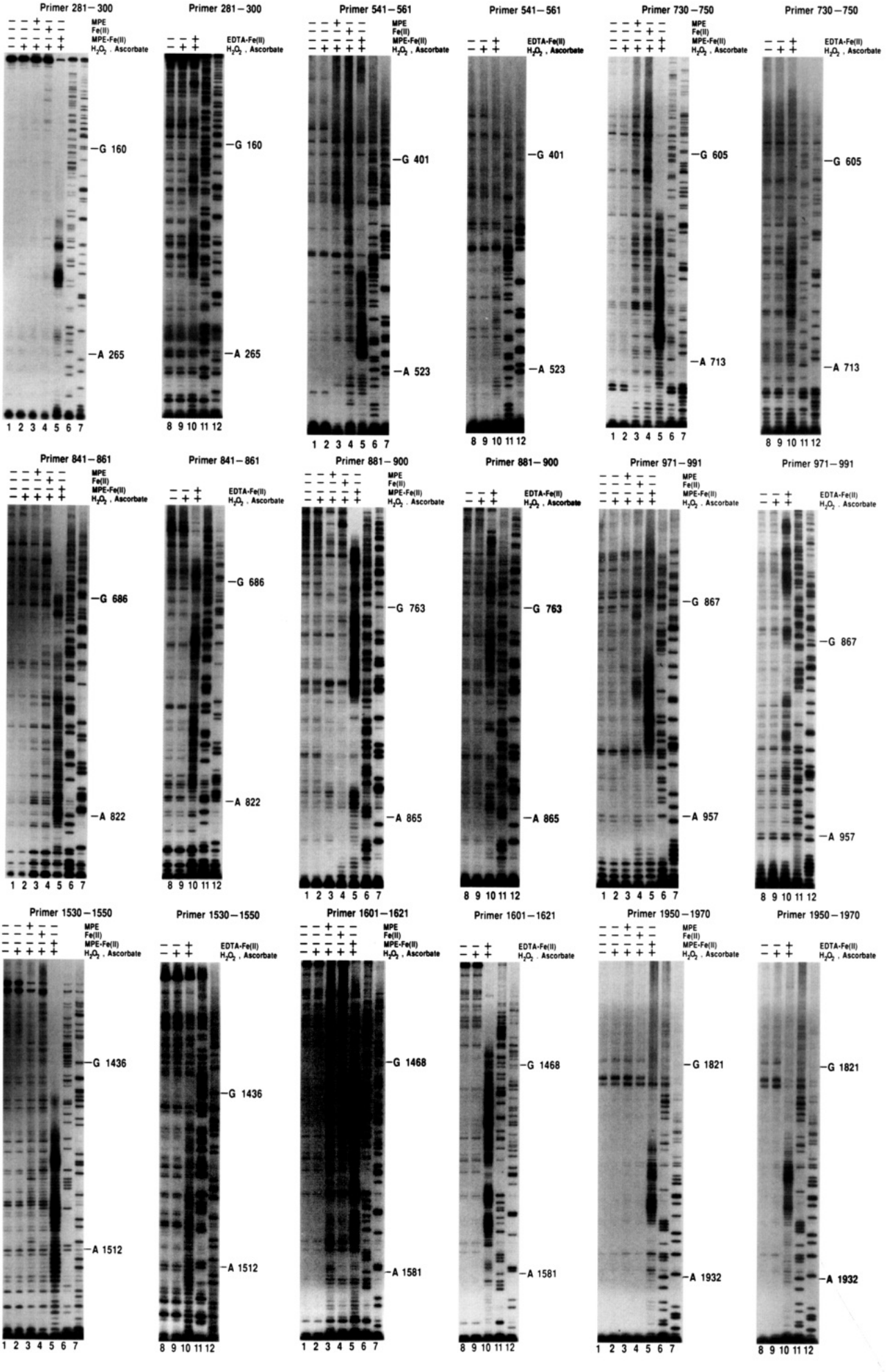
1	281 – 300	5'-AGATCTGTCGTCGGTACAAG-3'
2	430 – 450	5'-TGGGTAATTTACGCGCCTGCT-3'
3	541 – 561	5'-CCCTCCAATTGGTCCTTGTTA-3'
4	730 – 750	5'-ACAAGTATTTAATCACATATA-3'
5	841 – 861	5'-AAGCCTGCTTTAAGCACTCTA-3'
6	881 – 900	5'-TTATTTCATTATCCCATGCA-3'
7	971 – 991	5'-CACCTCTCGCGTCGTAATACT-3'
8	1030 – 1050	5'-TGAAAACATCTTTGGCAAATG-3'
9	1221 – 1245	5'-TGCCCTTCCGTCAATTCCTTTAAGT-3'
10	1330 – 1350	5'-CATAGATTCGAGAAAGAGCTA-3'
11	1530 – 1550	5'-GACAAACCAACAGGTACGGCT-3'
12	1601 – 1621	5'-CCTGTTATTGCTCAATCTCAT-3'
13	1781 – 1800	5'-ATGCGAGTTAATGACTCACA-3'
14	1909 – 1929	5'-AGTTCGGTCAACTTTTGCGAA-3'
15	1950 – 1970	5'-ACGGAAACCTTGTTACGACTT-3'

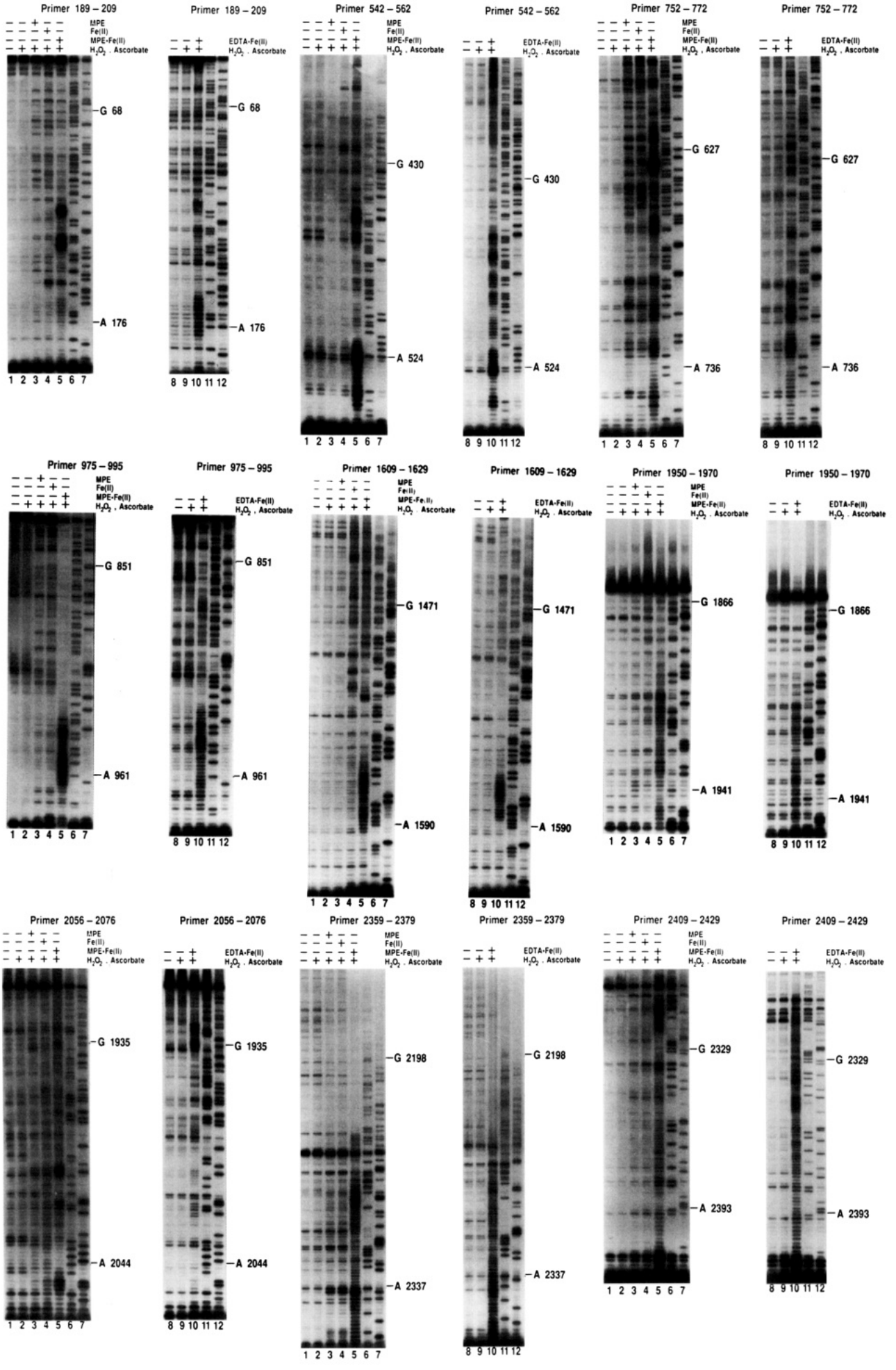
B. Primers for 28S rRNA

1	117 – 137	5'-AGACAAAGTGACTTAGTGCTG-3'
2	189 – 209	5'-AAGGACTTAAATCGTTAATTT-3'
3	344 – 364	5'-ACTATCGGTCTCATGGTTATA-3'
4	444 – 464	5'-CGGATATTCAGGTTCATCGGG-3'
5	542 – 562	5'-ATATGCTAATAGATTACAATG-3'
6	644 – 664	5'-AATCTATCAGCACTTTATCAA-3'
7	752 – 772	5'-CTGAATCTTTCGCATTGTTAA-3'
8	875 – 895	5'-AAATAGCTAAAAAACTAATCC-3'
9	975 – 995	5'-CATATATGCTCAAGGTACGTT-3'
10	1087 – 1107	5'-CTATACTCAATTCTGACAATC-3'
11	1187 – 1207	5'-CCAGATAAGATTATTTTATAT-3'
12	1287 – 1307	5'-CCTTGATCTTCATATCAAGAA-3'
13	1414 – 1434	5'-AAGCAACCAACGCCTTTCATG-3'
14	1546 – 1566	5'-ATCTACTTTAGCGGTAATGTA-3'
15	1609 – 1629	5'-GCATACCATTGTACCTTCCTA-3'
16	1758 – 1778	5'-TTAGGACCGACTAACTCGTGA-3'
17	1950 – 1970	5'-CTTCTTTATGGTCGTTCCTGT-3'
18	2056 – 2076	5'-ATATGTCATGCTCTTCTAGCC-3'
19	2167 – 2187	5'-AGACTCTTCACCTTGGAGACC-3'
20	2281 – 2301	5'-ATGTTATTGTTTCCCAATCAA-3'
21	2359 – 2379	5'-CGTAACTAAACTATCCGGGGA-3'
22	2409 – 2429	5'-CCCAAATAGTATTCTTAAAAA-3'
23	2468 – 2488	5'-AGTTCTGAATTGATTGTTAAT-3'
24	2548 – 2568	5'-AATCACATTGTGTCAACACCC-3'
25	2678 – 2698	5'-TAATCCATTCATGCGCGTCAC-3'
26	2783 – 2803	5'-TTAGAGTCAAGCTCAAAAGGG-3'
27	2853 – 2873	5'-GACGATACCAAACCGAGGTCT-3'
28	2982 – 3002	5'-ATCAAGAAGCTTGCATCAAAA-3'
29	3076 – 3096	5'-GGAGTTATACCAAATTTTCAA-3'
30	3139 – 3159	5'-GTACCGCCCCAGTCAAACTCC-3'
31	3289 – 3309	5'-TTTAAACCAAAAGGATCGATA-3'
32	3408 – 3428	5'-TCACAATGATAGGAAGAGCCG-3'
33	3518 – 3538	5'-CAACAACGTTTTGTCATTAGT-3'
34	3629 – 3649	5'-GTTCAGGCATAATCCAACGGA-3'
35	3717 – 3737	5'-TTATAAACTTTAAATGGTTTA-3'
36	3840 – 3860	5'-TGTCATTGTATTAAATAATGC-3'
37	3894 – 3914	5'-CCACTTACAACACCTTGCCTG-3'

FIGURE 2: DNA primers used for primer extension of *D. melanogaster* (A) 18S and (B) 28S rRNAs. The sequence is numbered according to Tautz et al. (1988). An additional 25 nucleotides of 28S rRNA absent in Tautz et al. (1988) are counted (Rousset et al., 1991).

were labeled at their 5′ ends by reaction with T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (Amersham) as described (Sambrook et al., 1989). Then 3 μL of 5′-end-labeled primers (~500 000 cpm) and 1 μL of 1 μg/μL RNA were dissolved in 1 μL of 5× hybridization buffer (250 mM K-Hepes, pH 7.0, 500 mM KCl). The reaction mixture was heated to 90 °C and then slowly cooled to 50 °C. The extension mixture was prepared by addition of 2 μL of 10× extension buffer (500 mM Tris-HCl, pH 8.3, 250 mM KCl, 100 mM $MgCl_2$), 4 μL of 2.5 mM dNTP (2.5 mM each dATP, dCTP, dGTP, and TTP), 0.5 μL of 80 mM DTT, 8 μL of H_2O, and 0.5 μL of AMV reverse transcriptase (~20 units/μL, Life Sciences) to 5 μL of the cooled RNA/primer hybrid mixture.


Primer 281–300
MPE
Fe(II)
MPE-Fe(II)
H2O2, Ascorbate
G 160
A 265
1 2 3 4 5 6 7
Primer 281–300
EDTA-Fe(II)
H2O2, Ascorbate
G 160
A 265
8 9 10 11 12
Primer 541–561
MPE
Fe(II)
MPE-Fe(II)
H2O2, Ascorbate
G 401
A 523
1 2 3 4 5 6 7
Primer 541–561
EDTA-Fe(II)
H2O2, Ascorbate
G 401
A 523
8 9 10 11 12
Primer 730–750
MPE
Fe(II)
MPE-Fe(II)
H2O2, Ascorbate
G 605
A 713
1 2 3 4 5 6 7
Primer 730–750
EDTA-Fe(II)
H2O2, Ascorbate
G 605
A 713
8 9 10 11 12
Primer 841–861
MPE
Fe(II)
MPE-Fe(II)
H2O2, Ascorbate
G 686
A 822
1 2 3 4 5 6 7
Primer 841–861
EDTA-Fe(II)
H2O2, Ascorbate
G 686
A 822
8 9 10 11 12
Primer 881–900
MPE
Fe(II)
MPE-Fe(II)
H2O2, Ascorbate
G 763
A 865
1 2 3 4 5 6 7
Primer 881–900
EDTA-Fe(II)
H2O2, Ascorbate
G 763
A 865
8 9 10 11 12
Primer 971–991
MPE
Fe(II)
MPE-Fe(II)
H2O2, Ascorbate
G 867
A 957
1 2 3 4 5 6 7
Primer 971–991
EDTA-Fe(II)
H2O2, Ascorbate
G 867
A 957
8 9 10 11 12
Primer 1530–1550
MPE
Fe(II)
MPE-Fe(II)
H2O2, Ascorbate
G 1436
A 1512
1 2 3 4 5 6 7
Primer 1530–1550
EDTA-Fe(II)
H2O2, Ascorbate
G 1436
A 1512
8 9 10 11 12
Primer 1601–1621
MPE
Fe(II)
MPE-Fe(II)
H2O2, Ascorbate
G 1468
A 1581
1 2 3 4 5 6 7
Primer 1601–1621
EDTA-Fe(II)
H2O2, Ascorbate
G 1468
A 1581
8 9 10 11 12
Primer 1950–1970
MPE
Fe(II)
MPE-Fe(II)
H2O2, Ascorbate
G 1821
A 1932
1 2 3 4 5 6 7
Primer 1950–1970
EDTA-Fe(II)
H2O2, Ascorbate
G 1821
A 1932
8 9 10 11 12


Primer 189 – 209
MPE
Fe(II)
MPE-Fe(II)
H2O2 . Ascorbate
G 68
A 176
1 2 3 4 5 6 7
Primer 189 – 209
EDTA-Fe(II)
H2O2 . Ascorbate
G 68
A 176
8 9 10 11 12
Primer 542 – 562
MPE
Fe(II)
MPE-Fe(II)
H2O2 . Ascorbate
G 430
A 524
1 2 3 4 5 6 7
Primer 542 – 562
EDTA-Fe(II)
H2O2 . Ascorbate
G 430
A 524
8 9 10 11 12
Primer 752 – 772
MPE
Fe(II)
MPE-Fe(II)
H2O2 . Ascorbate
G 627
A 736
1 2 3 4 5 6 7
Primer 752 – 772
EDTA-Fe(II)
H2O2 . Ascorbate
G 627
A 736
8 9 10 11 12
Primer 975 – 995
MPE
Fe(II)
MPE-Fe(II)
H2O2 . Ascorbate
G 851
A 961
1 2 3 4 5 6 7
Primer 975 – 995
EDTA-Fe(II)
H2O2 . Ascorbate
G 851
A 961
8 9 10 11 12
Primer 1609 – 1629
MPE
Fe(II)
MPE-Fe(II)
H2O2 . Ascorbate
G 1471
A 1590
1 2 3 4 5 6 7
Primer 1609 – 1629
EDTA-Fe(II)
H2O2 . Ascorbate
G 1471
A 1590
8 9 10 11 12
Primer 1950 – 1970
MPE
Fe(II)
MPE-Fe(II)
H2O2 . Ascorbate
G 1866
A 1941
1 2 3 4 5 6 7
Primer 1950 – 1970
EDTA-Fe(II)
H2O2 . Ascorbate
G 1866
A 1941
8 9 10 11 12
Primer 2056 – 2076
MPE
Fe(II)
MPE-Fe(II)
H2O2 . Ascorbate
G 1935
A 2044
1 2 3 4 5 6 7
Primer 2056 – 2076
EDTA-Fe(II)
H2O2 . Ascorbate
G 1935
A 2044
8 9 10 11 12
Primer 2359 – 2379
MPE
Fe(II)
MPE-Fe(II)
H2O2 . Ascorbate
G 2198
A 2337
1 2 3 4 5 6 7
Primer 2359 – 2379
EDTA-Fe(II)
H2O2 . Ascorbate
G 2198
A 2337
8 9 10 11 12
Primer 2409 – 2429
MPE
Fe(II)
MPE-Fe(II)
H2O2 . Ascorbate
G 2329
A 2393
1 2 3 4 5 6 7
Primer 2409 – 2429
EDTA-Fe(II)
H2O2 . Ascorbate
G 2329
A 2393
8 9 10 11 12

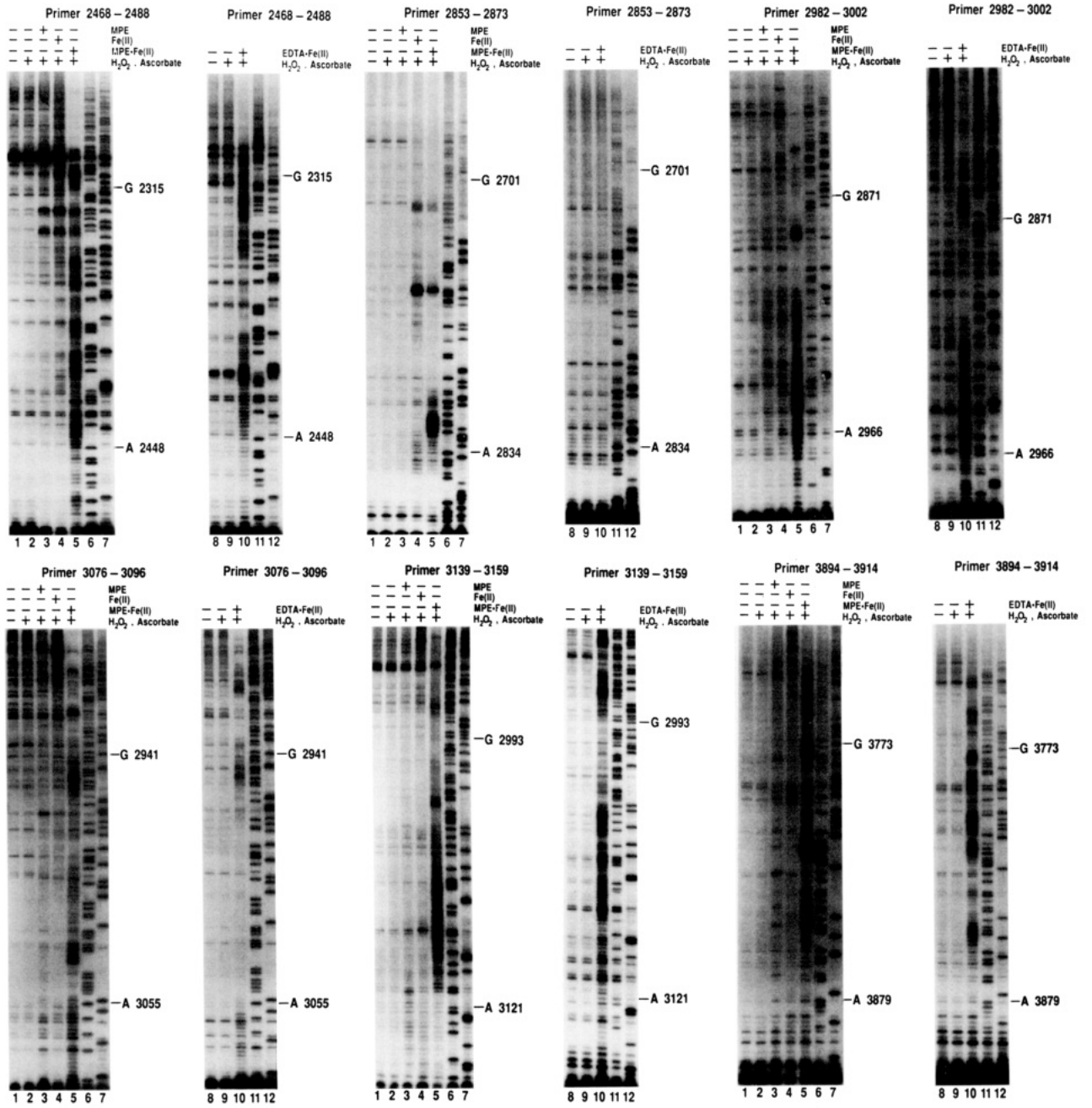
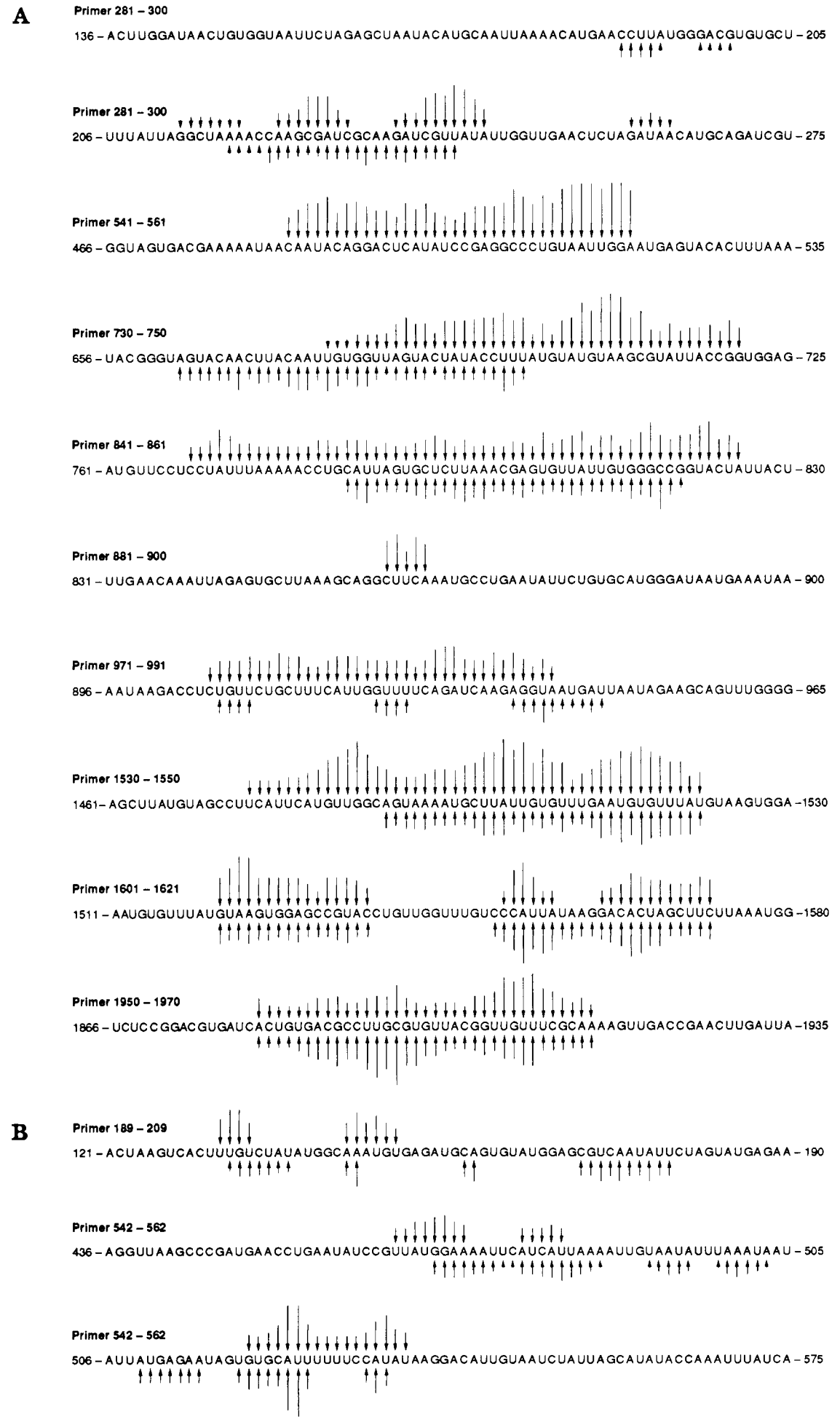
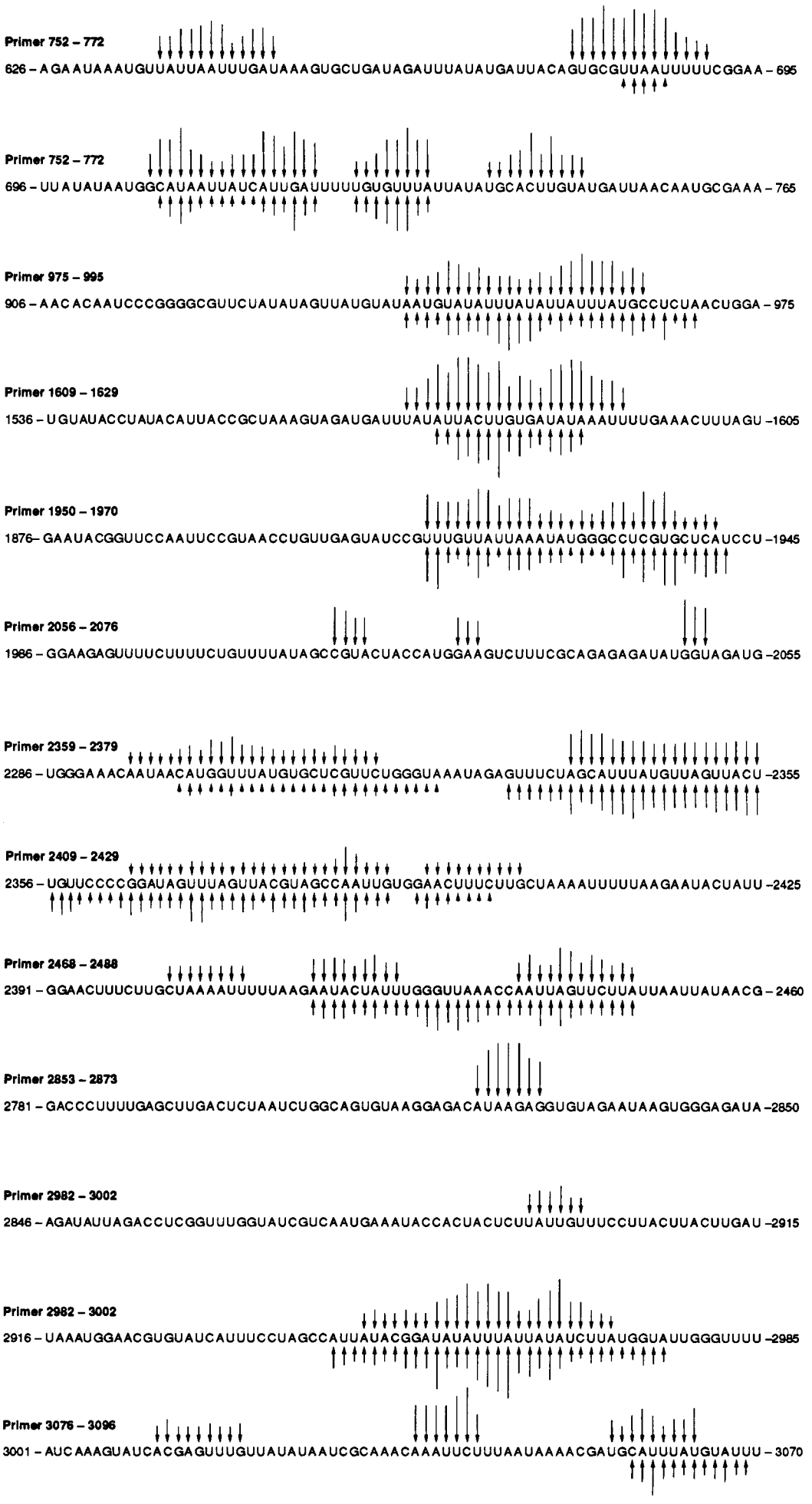


FIGURE 3: Autoradiograms of 8% denaturing polyacrylamide gels used to analyze the 5′-^{32}P-end-labeled DNA transcribed from *D. melanogaster* (first page) 18S and (second and third pages) 28S rRNA by primer extension using reverse transcriptase. (Lanes 1–5 and 8–10) DNA transcript products of reverse transcription of rRNAs prepared from ribosomes treated with various reagents: no reagent (lanes 1 and 8); H_2O_2 and sodium ascorbate (lanes 2 and 9); 50 μM MPE for 5 min, followed by 10 mM H_2O_2 and sodium ascorbate for 20 min (lane 3); 250 μM Fe(II) for 5 min, followed by 10 mM H_2O_2 and sodium ascorbate for 20 min (lane 4); 50 μM MPE·Fe(II) [50 μM MPE/250 μM Fe(II)], followed by 10 mM H_2O_2 and sodium ascorbate for 20 min (lane 5); 2.5 mM EDTA·Fe(II) [2.5 mM EDTA/2.5 mM Fe(II)], followed by H_2O_2 and sodium ascorbate for 20 min (lane 10). (Lanes 6, 7, 11, and 12) Dideoxy sequencing in the presence of ddT (lanes 6 and 11) or ddC (lanes 7 and 12) (Sanger et al., 1977).

Dideoxy sequencing reactions contained 2 μL of 2.5 mM dNTP and 2 μL of the appropriate 0.25 mM ddNTP instead of 4 μL of 2.5 mM dNTP (Sanger et al., 1977). Extension of the primer was allowed to proceed for 30 min at 43 °C and was terminated by addition of 100 μL of 0.3 M sodium acetate, pH 5.2, and 300 μL of 95% ethanol. The sample was cooled on ice for 1 h, and the cDNA transcripts were pelleted by centrifugation at 16000*g* for 20 min. The pellets were washed with 1 mL of 70% ethanol, dried under vaccum, and resuspended in 10 μL of loading buffer [80% formamide, 1× TBE (100 mM Tris–borate, pH 8.3, 2 mM EDTA), 0.02% bromophenol blue, 0.02% xylene cyanol]. Upon heat denaturation at 90 °C for 2 min followed by quick cooling, 100 000 cpm of the sample was loaded onto a wedge gel [$W \times L = 31.0$ cm × 38.5 cm, 0.4 mm at top and 1.2 mm at bottom, 8% polyacrylamide (1:20 Bis), 1× TBE, 7.5 M urea]. Electrophoresis was at 1200 V for 4–5 h until the bromophenol blue eluted from the bottom of the gel. The gel was transferred to Whatman 3MM paper and dried on a Bio-Rad Model 483 slab gel drier for 2 h at 80 °C. Kodak X-Omat AR film was exposed to the dried gel without an intensifying screen for 10–20 h at room temperature. Autoradiograms were scanned on a LKB UltroScan XL laser densitometer operating at 633 nm. Peak heights for each cleavage band were corrected by subtracting backgrounds from corresponding control lane bands and equated to the relative cleavage efficiencies at that site. These values are relative and cannot be compared with the values of the other gels.


A
Primer 281 – 300
136 – ACUUGGAUAACUGUGGUAAUUCUAGAGCUAAUACAUGCAAUUAAAACAUGAACCUUAUGGGACGUGUGCU – 205
Primer 281 – 300
206 – UUUAUUAGGCUAAAACCAAGCGAUCGCAAGAUCGUUAUAUUGGUUGAACUCUAGAUAACAUGCAGAUCGU – 275
Primer 541 – 561
466 – GGUAGUGACGAAAAAUAACAAUACAGGACUCAUAUCCGAGGCCCUGUAAUUGGAAUGAGUACACUUUAAA – 535
Primer 730 – 750
656 – UACGGGUAGUACAACUUACAAUUGUGGUUAGUACUAUACCUUUAUGUAUGUAAGCGUAUUACCGGUGGAG – 725
Primer 841 – 861
761 – AUGUUCCUCCUAUUUAAAAACCUGCAUUAGUGCUCUUAAACGAGUGUUAUUGUGGGCCGGUACUAUUACU – 830
Primer 881 – 900
831 – UUGAACAAAUUAGAGUGCUUAAAGCAGGCUUCAAAUGCCUGAAUAUUCUGUGCAUGGGAUAAUGAAAUAA – 900
Primer 971 – 991
896 – AAUAAGACCUCUGUUCUGCUUUCAUUGGUUUUCAGAUCAAGAGGUAAUGAUUAAUAGAAGCAGUUUGGGG – 965
Primer 1530 – 1550
1461 – AGCUUAUGUAGCCUUCAUUCAUGUUGGCAGUAAAAUGCUUAUUGUGUUUGAAUGUGUUUAUGUAAGUGGA – 1530
Primer 1601 – 1621
1511 – AAUGUGUUUAUGUAAGUGGAGCCGUACCUGUUGGUUUGUCCCAUUAUAAGGACACUAGCUUCUUAAAUGG – 1580
Primer 1950 – 1970
1866 – UCUCCGGACGUGAUCACUGUGACGCCUUGCGUGUUACGGUUGUUUCGCAAAAGUUGACCGAACUUGAUUA – 1935
B
Primer 189 – 209
121 – ACUAAGUCACUUUGUCUAUAUGGCAAAUGUGAGAUGCAGUGUAUGGAGCGUCAAUAUUCUAGUAUGAGAA – 190
Primer 542 – 562
436 – AGGUUAAGCCCGAUGAACCUGAAUAUCCGUUAUGGAAAAUUCAUCAUUAAAAUUGUAAUAUUUAAAUAAU – 505
Primer 542 – 562
506 – AUUAUGAGAAUAGUGUGCAUUUUUUCCAUAUAAGGACAUUGUAAUCUAUUAGCAUAUACCAAAUUUAUCA – 575


Primer 752 – 772
626 – AGAAUAAAUGUUAUUAAUUUGAUAAAGUGCUGAUAGAUUUAUAUGAUUACAGUGCGUUAAUUUUUCGGAA – 695
Primer 752 – 772
696 – UUAUAUAAUGGCAUAAUUAUCAUUGAUUUUUGUGUUUAUUAUAUGCACUUGUAUGAUUAACAAUGCGAAA – 765
Primer 975 – 995
906 – AACACAAUCCCGGGGCGUUCUAUAUAGUUAUGUAUAAUGUAUAUUUAUAUUAUUUAUGCCUCUAACUGGA – 975
Primer 1609 – 1629
1536 – UGUAUACCUAUACAUUACCGCUAAAGUAGAUGAUUUAUAUUACUUGUGAUAUAAAUUUUGAAACUUUAGU –1605
Primer 1950 – 1970
1876– GAAUACGGUUCCAAUUCCGUAACCUGUUGAGUAUCCGUUUGUUAUUAAAUAUGGGCCUCGUGCUCAUCCU –1945
Primer 2056 – 2076
1986 – GGAAGAGUUUUCUUUUCUGUUUUAUAGCCGUACUACCAUGGAAGUCUUUCGCAGAGAGAUAUGGUAGAUG –2055
Primer 2359 – 2379
2286 – UGGGAAACAAUAACAUGGUUUAUGUGCUCGUUCUGGGUAAAUAGAGUUUCUAGCAUUUAUGUUAGUUACU –2355
Primer 2409 – 2429
2356 – UGUUCCCCGGAUAGUUUAGUUACGUAGCCAAUUGUGGAACUUUCUUGCUAAAAUUUUUAAGAAUACUAUU –2425
Primer 2468 – 2488
2391 – GGAACUUUCUUGCUAAAAUUUUUAAGAAUACUAUUUGGGUUAAACCAAUUAGUUCUUAUUAAUUAUAACG – 2460
Primer 2853 – 2873
2781 – GACCCUUUUGAGCUUGACUCUAAUCUGGCAGUGUAAGGAGACAUAAGAGGUGUAGAAUAAGUGGGAGAUA –2850
Primer 2982 – 3002
2846 – AGAUAUUAGACCUCGGUUUGGUAUCGUCAAUGAAAUACCACUACUCUUAUUGUUUCCUUACUUACUUGAU –2915
Primer 2982 – 3002
2916 – UAAAUGGAACGUGUAUCAUUUCCUAGCCAUUAUACGGAUAUAUUUAUUAUAUCUUAUGGUAUUGGGUUUU –2985
Primer 3076 – 3096
3001 – AUCAAAGUAUCACGAGUUUGUUAUAUAAUCGCAAACAAAUUCUUUAAUAAAACGAUGCAUUUAUGUAUUU – 3070

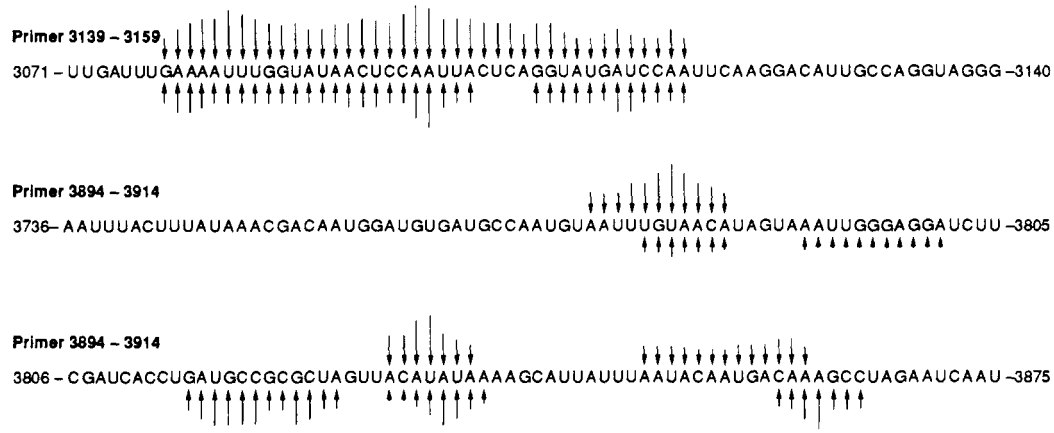


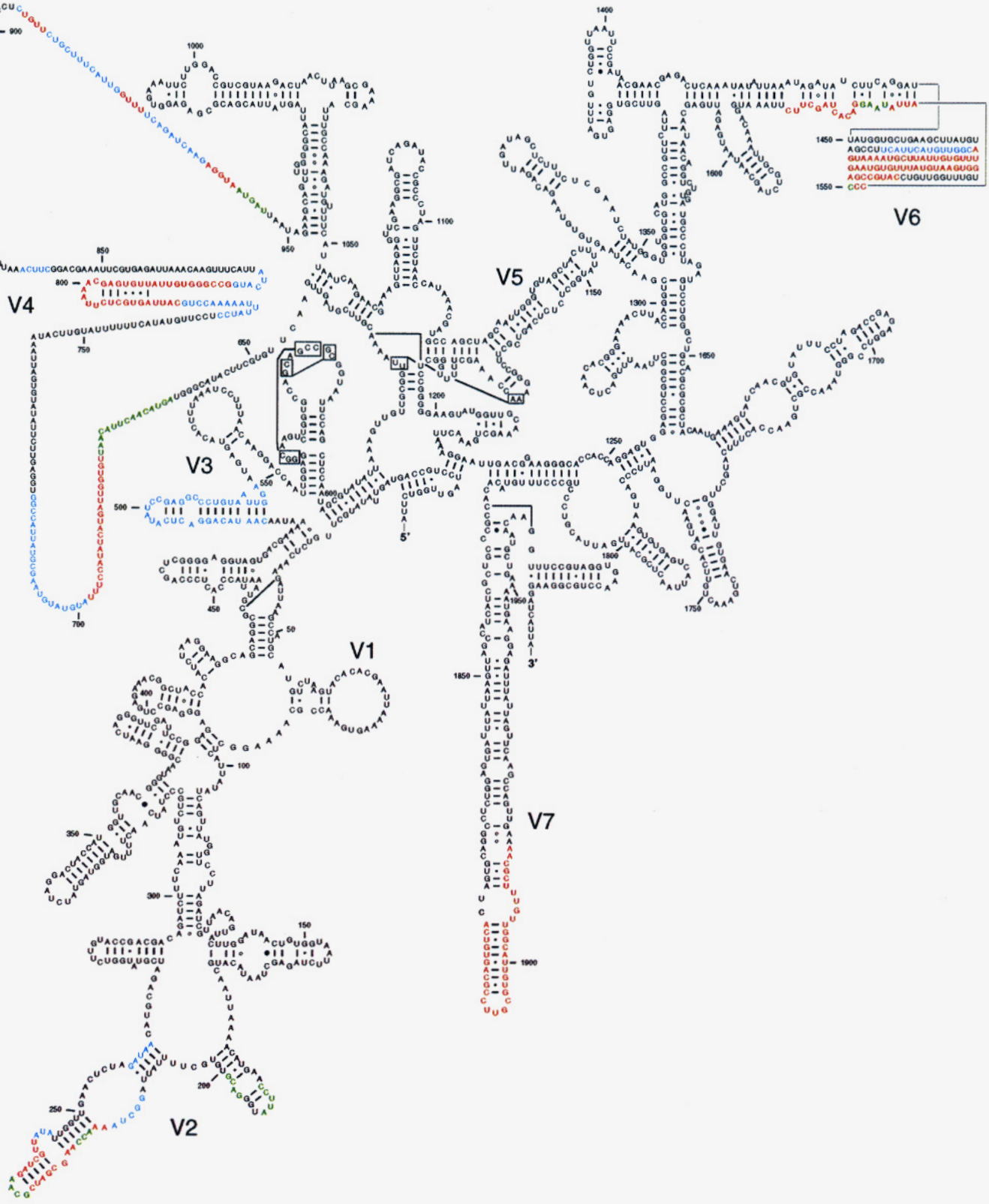
FIGURE 4: Histograms of the cleavage data derived from the autoradiograms in Figure 3: (A) 18S rRNA and (B) 28S rRNA. Cleavage sites by MPE·Fe(II) and EDTA·Fe(II) are indicated by arrows above and below the primary sequences, respectively. The heights of the arrows represent the relative cleavage intensities at the indicated bases.

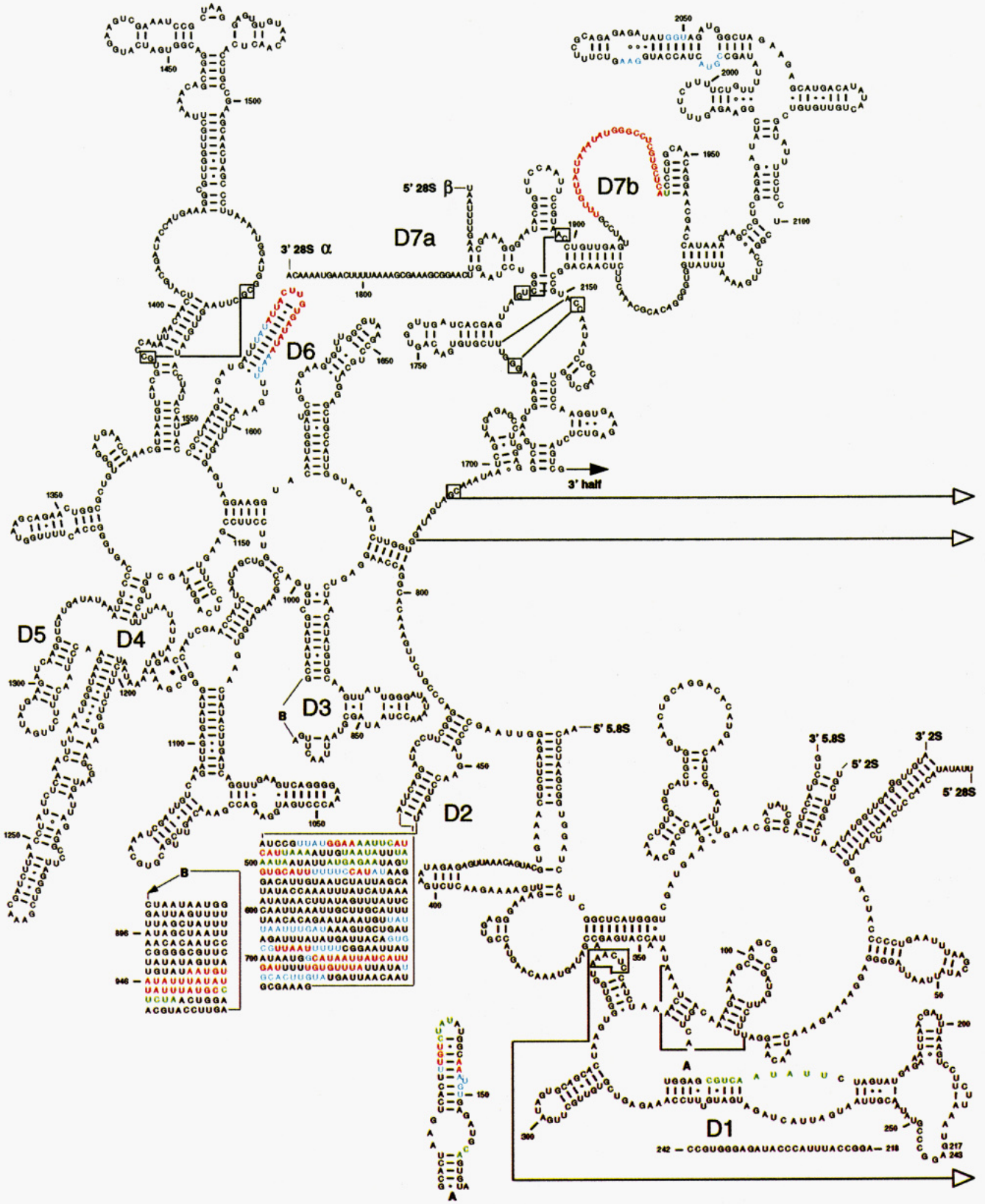
RESULTS AND DISCUSSION

Cleavage Reactions and Primer Extension. The scheme for cleavage of *D. melanogaster* ribosomes with MPE·Fe(II) or EDTA·Fe(II) is shown in Figure 1. *D. melanogaster* embryos were dechorionated and lysed on ice to afford the lysate. An *in vitro* translation experiment showed that the lysate was competent to convert poly(uridylic acid) into polyphenylalanine, indicating that ribosomes in the lysate were intact (unpublished data). The lysate was incubated with preformed MPE·Fe(II) or EDTA·Fe(II) for 5 min at room temperature, and the cleavage reaction was initiated with sodium ascorbate and hydrogen peroxide. After 30 min, total RNA was isolated using the guanidinium thiocyanate procedure (Chomczynski & Sacchi, 1987). Identification of the cleavage sites was performed by primer extension as described (Moazed et al., 1986; Stern et al., 1988b). The cleavage sites can be identified for distances of about 100–150 nucleotides from the priming position. A set of 15 and 37 DNA oligonucleotide primers have been used, covering the length from 1 to 1970 along the 18S rRNA (1995 nts in length, 1–1995) and from 1 to 3893 along the 28S rRNA (3925 nts in length, 1–1813 for 28Sα and 1859–3970 for 28Sβ), respectively (Figure 2) (Tautz et al., 1988; Rousset et al., 1991). The 5′-^{32}P-end-labeled primers were hybridized with the rRNA and extended with reverse transcriptase in the presence of the four deoxynucleoside triphosphates. The transcripts were analyzed by electrophoresis on an 8% denaturing polyacrylamide gel. All primers used for this study could be extended with reverse transcriptase in the presence of rRNA templates, confirming good selection of primers. Site-specific cleavage of the rRNA gave rise to bands corresponding to the length of DNA from the 5′ end of the primer to the nucleotide immediately preceding the 3′ side of the cleavage site. Cleavage sites were identified by comparison with dideoxy sequencing lanes run on the same gel. Artifact bands in the control lane, presumably arising from strong secondary structures in the RNA template or from nicks caused by the instabilty of template RNA compared to DNA, were distinguished from sites of MPE·Fe(II) or EDTA·Fe(II) attack by their occurrence in transcripts using the unreacted control RNA. The region between 1221 and 1279 of 18S rRNA could not be examined because the hypermodified nucleotide 3-(3-amino-3-carboxypropyl)-1-methylpseudouridine (amψ) at position 1279 halts reverse transcription of rRNA one nucleotide before amψ when 18S primer 10 (address 1330–1350) was used (Youvan & Hearst, 1981). The stop at base 1858 of 28S rRNA was seen because mature 28S rRNA in *D. melanogaster* consists of two fragments, 28Sα (1–1813) and 28Sβ (1859–3970) (Tautz et al., 1988). The extreme 3′ end of 18S, 28Sα, and 28Sβ could not be examined by primer extension (1950–1995 in 18S rRNA, 1758–1813 in 28Sα rRNA, and 3894–3970 in 28Sβ rRNA).

Cleavage Sites and Expansion Segments in 18S and 28S rRNAs. Autoradiograms from MPE·Fe(II) and EDTA·Fe(II) cleaving experiments are shown in Figure 3. The relative efficiency of cleavage at a site by MPE·Fe(II) was calculated densitometrically by subtracting the cleavage generated by 250 μM Fe(II) (lane 4) from that generated by 50 μM MPE·Fe(II) [50 μM MPE/250 μM Fe(II), a 1 to 5 molar ratio of MPE to Fe(II)] (lane 5). The relative efficiency of cleavage sites by EDTA·Fe(II) was calculated by subtracting the background control (lane 9) from cleavage generated by 2.5 mM EDTA·Fe(II) [2.5 mM EDTA/2.5 mM Fe(II), a 1 to 1 molar ratio of EDTA to Fe(II)] (lane 10). Histograms of MPE·Fe(II) and EDTA·Fe(II) cleavage sites are shown in Figure 4 (Tautz et al., 1988; Rousset et al., 1991). Only a limited number of sites in 18S and 28S rRNAs are cleaved by 50 μM MPE·Fe(II) and 2.5 mM EDTA·Fe(II) [323 nts (16%) and 229 nts (11%) in 18S rRNA and 431 nts (11%) and 422 nts (11%) in 28S rRNA by MPE·Fe(II) and EDTA·Fe(II), respectively]. Cleavage by MPE·Fe(II) occurs at a lower concentration than cleavage by EDTA·Fe(II) (50 μM vs 2.5 mM). In addition, MPE·Fe(II) induced cleavage at more sites than did EDTA·Fe(II) (16% vs 11% in 18S rRNA and 11% vs 11% in 28S rRNA). At a higher concentration of MPE·Fe(II) (500 μM), cleavage was more efficient, but no additional cleavage sites were observed.

The cleavage sites were mapped on the proposed secondary structure (Figures 5 and 6) (Hancock et al., 1988; Gutell, 1993; Gutell et al., 1993). The cleavage sites are clustered in a few distinct regions of secondary structure (V2–4 and V6–7 in 18S rRNA and D1–3, D6, D7b, stem 44, D8–10, and D12 in 28S rRNA). Most of the cleavage sites of 18S and 28S rRNA by MPE·Fe(II) and EDTA·Fe(II) at the concentrations used here are located within the expansion segments, rRNA elements with highly variable (V) or divergent (D) sizes and sequences, and not all expansion segments are cleaved (Clark et al., 1984; Stebbins-Boaz & Gerbi, 1991; Gerbi, 1994). Since MPE·Fe(II) and EDTA·Fe(II) require backbone accessibility for cleavage to occur, sites at which cleavage occurs are likely exposed on the surface of ribosomes. Cleavage is notably lacking in core segments, rRNA elements with highly conserved secondary structures, although the regions 2014–2017/2026–2028/2048–2050 and 2823–2829/2893–2898 in 28S rRNA are subject to cleavage


V1
V2
V3
V4
V5
V6
V7
5'
3'


D1
D2
D3
D4
D5
D6
D7a
D7b
5' 28S β
3' 28S α
3' half
5' 5.8S
3' 5.8S
5' 2S
3' 2S
5' 28S

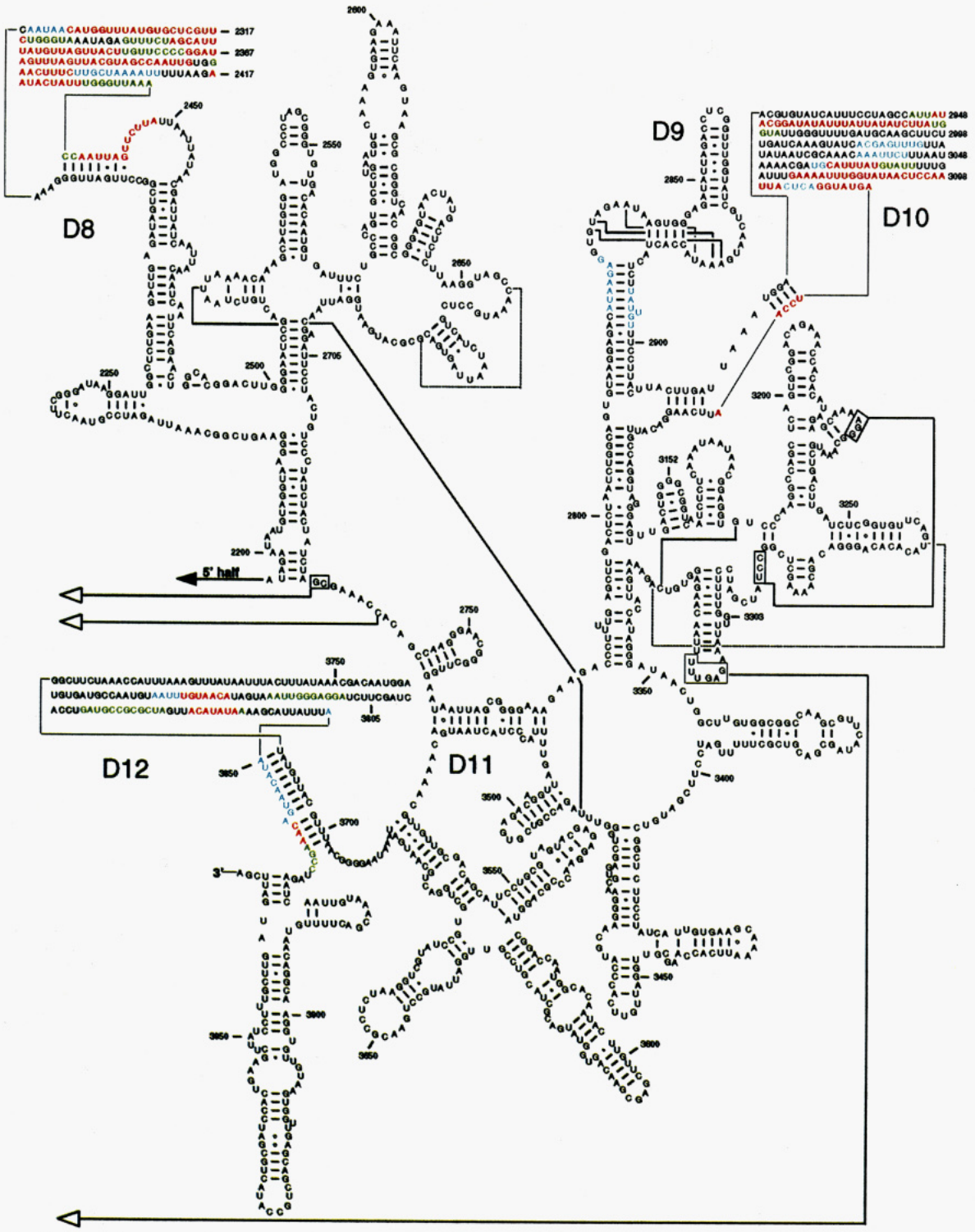


FIGURE 5: Cleavage sites mapped on the secondary structures of (A, first page) 18S and (B, second and third pages) 28S 5′-half and 3′-half rRNAs proposed by Gutell (Gutell, 1993; Gutell et al., 1993). An additional sequence of 25 nucleotides absent in Tautz et al. (1988) is presented between G217 and A243 and numbered from A218 to C242 in 28S rRNA (Rousset, 1991). Positions of core and expansion segments are indicated according to Hancock et al. (1988). Expansion segments are labeled V1–V7 for 18S rRNA and D1–D12 for 28S rRNA. Some expansion segments have not been modeled and are thus depicted as a block of nucleotides. The region between A1814 and A1858 is processed out of the 28S rRNA gene, yielding the 28Sα and 28Sβ rRNAs. Cleavage sites by both MPE·Fe(II) and EDTA·Fe(II), by only MPE·Fe(II), and by only EDTA·Fe(II) are shown in red, in blue, and in green, respectively.

by MPE·Fe(II). Lack of cleavage by MPE·Fe(II) and EDTA·Fe(II) is due to inaccessibility on the surface of fully assembled ribosomes caused by protection of rRNA with proteins, by involvement of rRNA in tertiary interactions with other portions of the rRNA, or by location of rRNA in interior regions of ribosomes.

A comparison of MPE·Fe(II) cleavage sites with those induced by EDTA·Fe(II) shows that most of the cleavage sites in 18S and 28S rRNAs are generated by both MPE·Fe(II) and EDTA·Fe(II) (Figures 4 and 5). However, several cleavage sites are generated exclusively by one of the reagents. For example, the V3 regions of 18S rRNA that are subject to cleavage by MPE·Fe(II) show almost no cleavage by EDTA·Fe(II). Therefore, this region of rRNA is somehow protected from cleavage by EDTA·Fe(II) but is still able to bind to MPE·Fe(II). This region might be a binding site for

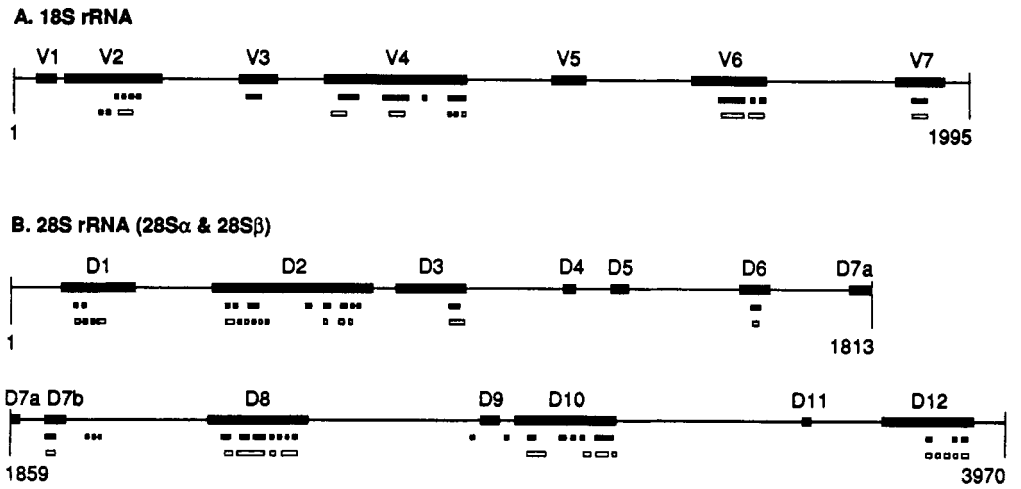


FIGURE 6: Schematic representation of (A) 18S and (B) 28S rRNAs. The positions of expansion segments are boxed in dark. Cleavage sites by MPE·Fe(II) and EDTA·Fe(II) are indicated by the black and white boxes, respectively.

loosely bound proteins, which can be displaced by the intercalator MPE·Fe(II) or exposed by induced conformational changes in rRNA upon binding of MPE·Fe(II) to other rRNA sites.

With a few exceptions, most of cleavage sites of 18S and 28S rRNAs by MPE·Fe(II) occur in double-stranded stem regions and at junctions between stems and loops [e.g., see V2 (207–263), V3 (484–519), and V7 (1881–1915) in 18S rRNA and D1 (127–152), D6 (1567–1592), and core segments (2010–2060 and 2823–2898) in 28S rRNA (Figure 5)]. This is in general agreement with an intercalative binding mode for MPE·Fe(II). A similar result was observed for a 345-nt fragment of *E. coli* 16S rRNA (Kean et al., 1985). This suggests that the cleavage pattern by MPE·Fe(II) could be used to distinguish different secondary structures of equivalent energy. Two possible secondary structural models which cannot be distinguished on energetic criteria have been proposed for the 830–946 region of the 18S rRNA expansion segment V4 by Hancock et al. (1988) (models A and B in Figure 7). The alternative potential secondary structure for this region was proposed by De Rijk et al. (1992) (Figure 7C). Superposition of our cleavage data on these structures of the 830–946 region reveals that the cleavage sites on structure A are located in stems and at stem–loop junctions, while in structure B the sites are located in several unstructured regions. Thus, this region is most likely represented by structure A, as shown in Figure 7A (Hancock et al., 1988). Model C also seems to fit the experimental cleavage data for the 830–946 region (Figure 7C) (De Rijk et al., 1992). We cannot rule out the possibility that tertiary interactions also could account for the cleavage data.

The presence of certain surface-exposed rRNA sequences in expansion segments might be important evidence for morphological structures of eukaryotic ribosomes. Eukaryotic small ribosomal subunits contain structural features termed eukaryotic lobes and archaebacterial bills at the bottom and on the head of the subunit, respectively (Henderson et al., 1984; Oakes et al., 1986). Eukaryotic lobes of small ribosomal subunits are composed primarily of rRNA and thought to be about 300 nucleotide long (Kuhlbrandt & Unwin, 1982). Three-dimensional models of *E. coli* small ribosomal subunits predict that the eukaryotic lobes and archaebacterial bills consist of expansion segments of eukaryotic 18S rRNA (Brimacombe et al., 1988; Stern et al., 1988). On the basis of the model, expansion segments V2, V4, and V7 in *D. melanogaster* 18S rRNA are related to eukaryotic lobes and V6 constitutes archaebacterial bills. In addition, these expansion segments can be accommodated into the compact structure of the *E. coli* small ribosomal subunit by being exposed on the ribosome surface. Indeed, these expansion segments were cleaved by MPE·Fe(II) and EDTA·Fe(II) [206 MPE·Fe(II) and 157 EDTA·Fe(II) cleavage sites for V2, V4, and V7; 81 MPE·Fe(II) and 72 EDTA·Fe(II) cleavage sites for V6]. These results provide experimental evidence that eukaryotic lobes as well as archaebacterial bills are composed primarily of accessible rRNA and suggest which sequences are contained within them. Eukaryotic lobes are also present at the bottom of the eukaryotic large ribosomal subunit and are exposed on the surface (Henderson et al., 1984; Oakes et al., 1986). It is possible that sequences in expansion segments accessible to these cleaving reagents in 28S rRNA might be involved in the eukaryotic lobe structure of the large subunit.

A genetic tag has been inserted within eukaryotic 26S rRNAs for analysis of eukaryotic ribosomal mutations. The 18 and 119 base-pair tags that were inserted within the first expansion segment of domain I of *Saccharomyces cerevisiae* 26S rRNA and the D12 segments of rRNA of *Tetrahymena thermophila* 26S rRNA, respectively, were not deleterious to the cells, suggesting that the biosynthesis and function of ribosomes are not affected by such insertions (Musters et al., 1989; Sweeney & Yao, 1989). These sites correspond to the D1 and D12 expansion segments of *D. melanogaster* where MPE·Fe(II) cleavage sites were observed (Figure 5). These results suggest that MPE·Fe(II) and EDTA·Fe(II) cleavage sites could provide information about the other sites within rRNA that are good candidates for the insertion of a genetic tag. The insertion of a tag within MPE·Fe(II) and EDTA·Fe(II) cleavage sites would likely not interfere with the proper processing, assembly, and functioning of ribosomes.

Cleavage patterns on expansion segments might be used for designing oligonucleotides of potential value for eukaryotic ribosomal RNA-based cell staining, immune electron microscopic studies, and the preparation of heavy-metal derivatives of ribosomes for the phase evaluation of crystallographic studies (DeLong et al., 1989; Oakes & Lake, 1990; Oakes et al., 1990; Weinstein et al., 1989; Yonath & Wittmann, 1989; Lasater et al., 1988; Hill et al., 1990). Because of the abundance of rRNA in cells and the high sequence variability of the expansion segments among species, phylogenetic, species-specific, fluorescently labeled oligonucleotides complementary to the putative single-stranded region of surface-exposed rRNA in expansion segments might be useful to distinguish between intact eukaryotic cells and to measure the ribosome content of different cell types (DeLong et al.,

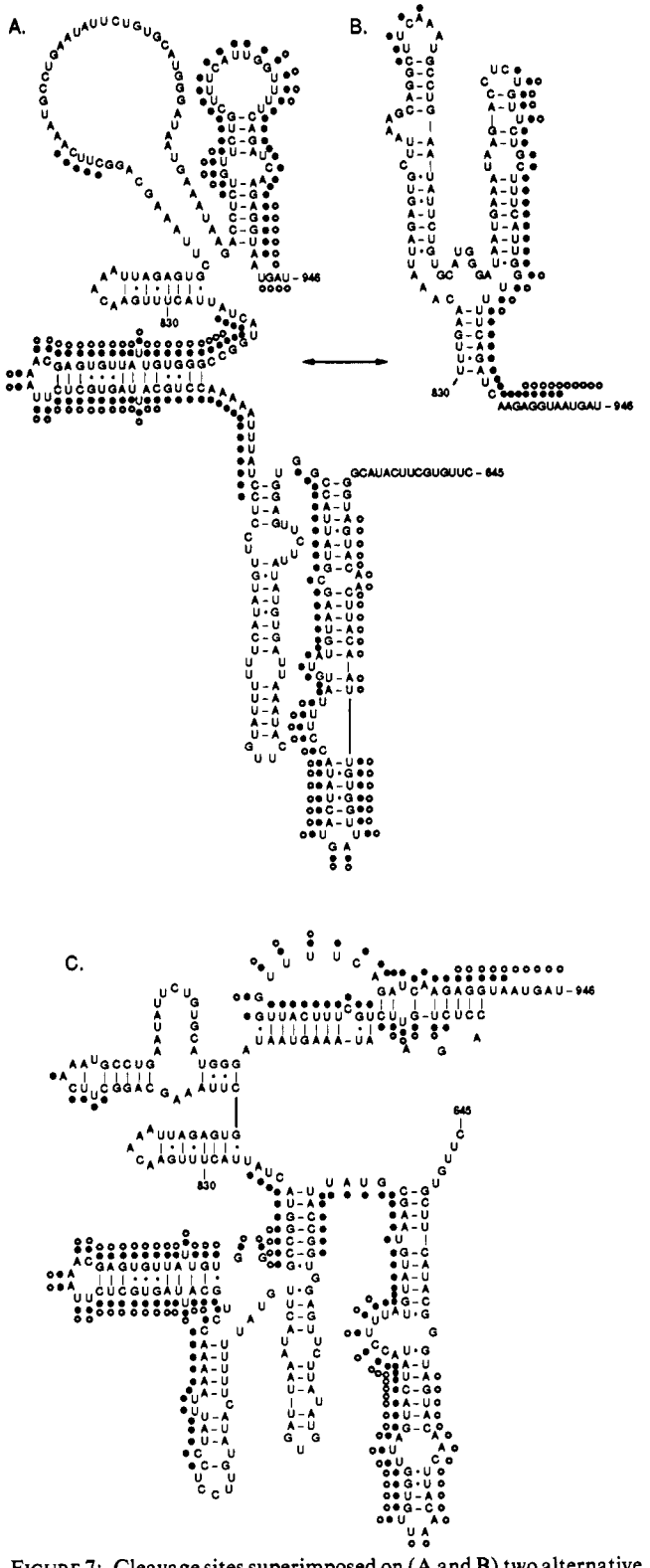


FIGURE 7: Cleavage sites superimposed on (A and B) two alternative secondary structures of equivalent energy and (C) the other model proposed by Hancock et al. (1988) and De Rijk et al. (1992), respectively, in the region located between positions 830 and 946 of the 18S rRNA V4 domain. Filled and open circles indicate cleavage sites by MPE·Fe(II) and EDTA·Fe(II), respectively. Cleavage data favor models A and C for the 830–946 region.

1989). These probes should hybridize with rRNA in expansion segments of intact ribosomes since cleavage sites are presumably exposed on the surface of eukaryotic ribosomes so that the delivery of probes is thought to be less hindered. The availability of specific rRNA sequences on the surface of eukaryotic ribosomes allows these regions to be mapped on the three-dimensional structure of ribosomes by using DNA-hybridization electron microscopy (Oakes & Lake, 1990; Oakes et al., 1990). Surface-exposed single-stranded rRNA in expansion segments can be a target site for introduction of a heavy-atom compound to ribosomes using DNA oligonucleotides (Weinstein et al., 1989; Yonath & Wittmann, 1989).

CONCLUSIONS

In conclusion, a combination of MPE·Fe(II) and EDTA·Fe(II) can be used to identify, to nucleotide resolution, regions of rRNAs that are accessible on the surface of the eukaryotic ribosomes in an actively translating *in vitro* system. Generation of a nonspecific diffusible oxidant by MPE·Fe(II) and EDTA·Fe(II) is not inhibited by redox-inactive metal ions present in the cell-free *in vitro* system. The application of this technique to intact ribosomes from other eukaryotic species will provide information about the conservation of ribosomal RNA packaging within ribosomes and be useful for studies of the evolutionary origins and phylogenetic investigations of expansion segments from different species of eukaryotes. In addition, information about the cleavage sites may be useful in the aspect of designing experiments required to study eukaryotic ribosomes.

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