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RNase MRP and RNase P share a common substrate

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ABSTRACT

RNase MRP is a site-specific ribonucleoprotein endoribonuclease that processes RNA from the mammalian mitochondrial displacement loop containing region. RNase P is a site-specific ribonucleoprotein endoribonuclease that processes pre-tRNAs to generate their mature 5′-ends. A similar structure for the RNase P and RNase MRP RNAs and a common cleavage mechanism for RNase MRP and RNase P enzymes have been proposed. Experiments with protein synthesis antibiotics have shown that both RNase MRP and RNase P are inhibited by puromycin. We also show that *E.coli* RNase P cleaves the RNase MRP substrate, mouse mitochondrial primer RNA, exactly at a site that is cleaved by RNase MRP.

INTRODUCTION

RNase MRP is a site specific ribonucleoprotein (RNP) endoribonuclease that processes RNA from the mammalian mitochondrial displacement loop (D-loop) containing region at multiple, discrete sites *in vitro* (1, 2). As very few RNase MRP RNA copurifies with mitochondria (3), the substrate specificity is considered the strongest support for a mitochondrial function (4). However, RNase MRP is a nuclear enzyme (2), but no nuclear substrate for RNase MRP has been described although it is highly likely to exist (5–7). RNase P is the ubiquitous site specific RNP endoribonuclease that processes pre-tRNAs to generate their mature 5′-termini (8).

Both RNase MRP and RNase P cleave RNA to generate 5′-phosphate and 3′-hydroxyl termini in a divalent cation dependent manner (1, 8). The RNA components of both human RNAse MRP and RNase P RNA share some limited sequence similarities (9). A similar cage or core structure for the RNA components of RNase P and RNase MRP enzymes and a common cleavage mechanism have been proposed (10). A recent phylogenetic comparison of RNase MRP RNAs showed that they can indeed be folded into an RNase P RNA like structure (11). Furthermore, the vertebrate RNase P and RNase MRP enzymes share protein components (12) and they are associated with the Th/ To RNP (13), suggesting interrelated pathways and/ or functions (2, 9–18).

We have extended the analysis on the relationship between RNase MRP and RNase P. We have employed the antibiotic puromycin, a specific inhibitor of *E.coli* RNase P (19). We have also tested if *E.coli* RNase P is able to process RNA from the mitochondrial (mt) D-loop containing region, D-loop mtRNA, a substrate for RNase MRP.

MATERIAL AND METHODS

Radioactivity was purchased from N.E.N. Puromycin-dihydrochloride was purchased from U.S.B. and cycloheximide from Sigma. 5′-end labeled DNA molecular weight marker VIII (Boehringer) was used as a molecular weight standard for denaturing gel electrophoresis.

Preparation of RNA substrates

Plasmids. pMR718B for transcription of D-loop mtRNA has been described (1). pUC19/ pTyr was digested with FokI prior to transcription and contains the sequence for the *E.coli* pre-tRNA^{Tyr} behind a T7 RNA polymerase promotor. pM1-8 contains the gene for M1 RNA cloned into the EcoRI/ XbaI sites of the vector pGem1 by genomic amplification (20) with primers that added an EcoRI site directly to the 5′-end and a XbaI site directly to the 3′-end of the gene. The M1 RNA transcribed from this vector contains the additional 5′- and 3′-polylinker sequence from the pGem1 vector and the A to G transition at position +96 (21).

Transcriptions. In vitro transcriptions were done essentially as described (20).

3′-end labeling of RNAs. 3′-end labeling of RNAs was carried out as described (1) with the exception that the RNAs were gel purified on a small 6% polyacrylamide/7 M urea gel prior to the labeling reaction. RNA bands were visualized by Ethidium bromide staining and eluted as described (1).For quantitation of the 3′-end labeling reaction 2 μg of substrate RNA (determined by spectrophotometric analysis) were labeled with [^{32}P]pCp as described. Then the ratio of incorporated versus total radioactivity was determined by liquid scintillation counting. Incorporation of radioactivity was determined by 4 times precipitation with trichloroacetic acid (20) or with equal results by 3×precipitation with ethanol in the presence of 2,5 M ammonium acetate (20).

Preparation of enzymes

Preparation of E.coli RNase P. Crude RNase P preparations were prepared essentially as described (22): *E.coli* DH5 alpha was grown in 1000 ml of LB at 37°C to an OD_{600} of 0.6. Cells were harvested, washed and resuspended in 5 ml of buffer A (50 mM

Tris−HCl pH 7.5, 10 mM $MgCl_2$, 60 mM KCl, 10% glycerol and 1 mM DTT). The cells were disrupted by sonication with a Branson sonifier. Cell debris was removed by centrifugation in a TLA 100.2 rotor for 15 minutes at 29000 rpm. The cleared S-30 extract was then centrifuged in the same rotor at 53000 rpm for 70 minutes. The pellet was dissolved in 4 ml of buffer A plus 500 mM KCl. The extract was then dialyzed against 500 ml of buffer B (20 mM Hepes-KOH pH 7.6, 10% glycerol, 50 mM KCl, 0.5 mM EDTA and 1 mM DTT). For further purification 1 ml of this extract was concentrated by Centricon 30 (Amicon) centrifugation to a final volume of 200 μl and loaded on a 15% to 30% glycerol gradient containing 20 mM Hepes-KOH pH 7.6, 150 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA and 1 mM DTT and centrifuged in a SW41Ti rotor for 24 hrs at 34000 rpm. Fractions of about 600 μl were collected and assayed for RNase P activity. Active fractions were pooled, concentrated by Centricon 30 centrifugation and loaded on a second gradient. Again fractions of about 600 μl were collected, assayed for RNase P activity, frozen in liquid nitrogen and stored at −80°C.

Preparation of mouse RNase P and RNase MRP. RNase P and RNase MRP were purified by glycerol gradient centrifugation from mouse LA9 cell nuclear extract essentially as described (2). Gradient fractions that contained both RNase MRP and RNase P activity were used throughout this study.

RNA processing reactions

In vitro RNA processing. For RNA only reactions M1 RNA (gelpurified, 500 ng/assay) was incubated in a buffer containing 50 mM Hepes-KOH pH 7.6, 100 mM NH_4Cl, and 100 mM $MgCl_2$ (standard reaction buffer R1) with 10000 cpm of substrate RNA in a total volume of 10 μl for one hour at 37°C. Then the RNAs were precipitated with 20 μg glycogen (Boehringer) as carrier and analyzed on 6% polyacrylamide/7 M urea gels. For reactions with *E.coli* RNase P, mouse RNase MRP and mouse RNase P, 2 μl of a glycerol gradient fraction were incubated in a buffer containing 50 mM Hepes-KOH pH 7.6, 40 mM KCl, 10 mM $MgCl_2$ and 1 mM DTT (standard reaction buffer R2) with 10000 cpm of substrate RNA in a total volume of 20 μl for 30 minutes at 37°C. The reactions were stopped by extraction with Phenol/Chloroform/Isoamylalcohol (25:24:1), the RNA was precipitated with 20 μg glycogen (Boehringer) as carrier and analyzed on 6% polyacrylamide/7 M urea gels.

Pretreatment of E.coli RNase P with micrococcal nuclease. 2 μl RNase P fraction were preincubated in a 4 μl volume with 1 mM $CaCl_2$, or 1 mM $CaCl_2$ plus 25 mM EGTA, or 25 mM EGTA or with micrococcal nuclease (0.05−0.2 units/μl) in the presence of 1 mM $CaCl_2$ for 10 minutes at 37°C. The fractions that had been preincubated with micrococcal nuclease (0.05−0.2 units/μl) were then adjusted to 25 mM EGTA (pH 8.0) to inactivate the micrococcal nuclease. Control fractions received increasing amounts of pre-inactivated (25 mM EGTA) micrococcal nuclease (0.05−0.2 units/μl) or 0.05 units/μl micrococcal nuclease alone (to check the activity of the micrococcal nuclease towards the mouse D-loop mtRNA). Finally substrate RNA was added and the reaction was adjusted to reaction buffer R2 conditions in a final volume of 20 μl; incubation was continued for one hour at 37°C.

Antibiotic inhibition of RNase P and RNase MRP. Puromycin-dihydrochloride was dissolved in water and neutralized with NaOH to obtain a 50 mM puromycin/100 mM NaCl stock solution. 2 μl of a glycerol gradient fraction containing RNase MRP and RNase P were preincubated with the antibiotic in a buffer containing 50 mM Hepes pH 7.6, 40 mM NaCl, 10 mM $MgCl_2$ and 1 mM DTT (standard reaction buffer R3) for 5 minutes at 37°C. As the concentration of NaCl has some influence on the processing reaction and the puromycin stock solution contains NaCl all assays were adjusted to a final NaCl concentration of 40 mM. The reaction was started by the addition of 10000 cpm of 3′-end labeled RNA and was incubated for 30 minutes at 37°C.

Quantitative analysis of the RNase P reactions. Each experiment was performed three times with independently labeled and quantitated RNA. About 17,5 fmol substrate RNA per 20 μl assay were used.

For the determination of the relative molar cleavage efficiency of D-loop mtRNA processing and precursor $tRNA^{Tyr}$ processing different amounts of *E.coli* RNase P were incubated with substrate RNA under the appropriate standard reaction conditions for 1 hour. The relative cleavage efficiency of the reactions was determined by analysing the gels on a PhosphorImager (Molecular Dynamics). A rectangle was laid over each lane and the area integration mode and the baseline bestfit option were used.

RNA analysis. RNase T1 digestion and partial alkaline hydrolysis of end labeled RNA were performed as described (23). The products were analyzed on 8% polyacrylamide/7M urea gels.

RESULTS

Both RNase MRP and RNase P are sensitive to puromycin

It was reported that M1 RNA catalyzed pre-tRNA processing is specifically inhibited by the protein synthesis antibiotic puromycin; *E.coli* RNase P holoenzyme can be inhibited by similar concentrations of puromycin (19). The RNA components of RNase MRP have been proposed to form a similar cage or core structure as the RNA components of RNase P enzymes (10, 11). Because one could expect closely related enzymes to respond in a similar fashion to antibiotics we tested if mouse RNase MRP and RNase P can be inhibited by puromycin. We employed glycerol gradient fractions containing RNase MRP and RNase P with the antibiotic inhibitor puromycin (Figure 1). Increasing concentrations of puromycin were preincubated with constant amounts of RNase P and RNase MRP and then employed in standard assays with D-loop mtRNA (Figure 1a) or pre-$tRNA^{Tyr}$ (Figure 1b) as described in Materials and Methods. Interestingly, both activities are sensitive to puromycin whereas the control antibiotic cycloheximide has no effect in the concentration range tested. Employing standard assay conditions (buffer R3), inhibition of D-loop mtRNA processing was apparently complete at 5 mM of puromycin, whereas pre-$tRNA^{Tyr}$ processing was apparently not inhibited at this concentration of puromycin. Inhibition of pre-$tRNA^{Tyr}$ cleavage was apparently complete at 8 mM of puromycin, employing standard assay conditions (buffer R3).

Since it has been proposed (2) that RNase MRP processes D-loop mtRNA in a 5′ to 3′ fashion it could be possible that only

Figure 1. Puromycin inhibits mouse RNase MRP and RNase P. 3′-end labeled mouse D-loop mtRNA or *E.coli* pre-tRNA^{Tyr} (pTyr) were incubated with standard reaction buffer R3 without enzyme (mock). For antibiotic inhibition, fractions containing both RNase MRP and RNase P were preincubated with increasing amounts of puromycin or cycloheximide and the reactions were started by adding substrate RNA as described in Materials and Methods. The pTyr and 5′-end processed product are indicated at the left. The D-loop mtRNA substrate, as well as the cleavage products, are indicated at the left with conserved sequence blocks I, II, and III depicted in boxes (2).

Figure 2. Processing of 3′-end labeled mouse D-loop mtRNA by RNase MRP, M1 RNA and *E.coli* RNase P. Standard assays were carried out as described in Materials and Methods. As reference a partial alkaline hydrolysis ladder (OH$^-$) showing all residues and an RNase T1 digest (T1) showing G residues were used. The mouse RNase MRP cleavage positions (site 1 and site 2) and the *E.coli* RNase P as well as M1 RNA cleavage position (identical with site 2) are indicated. The site 3 cleavage product of RNase MRP has run out of the gel.

the first cleavage site (site 1) of RNase MRP is inhibited by puromycin and that therefore the subsequent cleavages at site 2 and site 3 are inhibited as well. To test this possibility directly, site 1 and site 2 cleavage products of an RNase MRP assay were cut out from a gel, eluted, and used as substrates for RNase MRP in the presence of varying puromycin concentrations (data not shown). Using site 1 cleavage product as the substrate, small amounts of the other cleavage products were observed, as described previously (2). Processing of the site 1 cleavage product at site 2 and site 3 is still sensitive to puromycin. Using site 2 cleavage product as the substrate, no site 3 cleavage product was observed. Thus processing at site 2 and site 3 is inhibited by puromycin independent from cleavage at site 1.

The inhibition of both RNase P and RNase MRP by similar concentrations of puromycin indicates that the enzymes share some common feature.

E.coli RNase P cleaves D-loop mtRNA at an RNase MRP cleavage site

Apparently, RNase MRP and RNase P are closely related enzymes, but in the absence of RNase P, RNase MRP does not cleave a tRNA precursor (2, 17). We wanted to know if RNase P can cleave the RNase MRP substrate, D-loop mtRNA. We purified RNase P from crude extracts of *E.coli* cells by successive glycerol gradient centrifugation. The RNase P peak fraction was used for assays with the the best characterized RNase MRP substrate, the mouse D-loop mtRNA (1, 2). Interestingly, the mouse D-loop mtRNA is a substrate for the *E.coli* activity: the activity that copurifies with *E.coli* RNase P cleaves mouse D-loop mtRNA, exactly at the cleavage site 2 of RNase MRP (Figure 2). The molar cleavage efficiency of RNase P with D-loop mtRNA was determined relative to pre-tRNA^{Tyr} processing. Serial diluted amounts of RNase P were incubated with equal molar amounts of D-loop mtRNA and pre-tRNA^{Tyr}. The relative cleavage efficiencies of both substrates could then be estimated by comparing the reactions manifesting roughly the same level of activity. By this method cleavage of D-loop mtRNA

by *E.coli* RNase P is about 250 times less efficient than cleavage of pre-tRNA^{Tyr} (data not shown). No cleavage of D-loop mtRNA by *E.coli* RNase P at the cleavage sites 1 and 3 of RNase MRP was observed.

We wanted to know if the *E.coli* D-loop mtRNA processing activity is sensitive puromycin. In fact, increasing concentrations of puromycin specifically inhibited the cleavage of the mouse D-loop mtRNA by the *E.coli* RNase P (apparently complete inhibition occurred at around 5 mM) whereas the control antibiotic cycloheximide had no effect.(concentrations up to 10 mM, data not shown). Moreover, pretreatment of the enzyme fraction with micrococcal nuclease leads to inactiviation of 'site 2' cleavage (Figure 3). This result suggests that cleavage of D-loop mtRNA by the *E.coli* activity at this site requires an RNA component.

To directly test the involvement of RNase P in D-loop mtRNA processing at site 2 we prepared M1 RNA, the catalytic subunit of *E.coli* RNase P (8), by *in vitro* transcription and purification on denaturing gels as described in Materials and Methods. Figure 2 shows that the catalytic RNase P RNA cleaves D-loop mtRNA exactly at this site. The cleavage product migrates slightly different than the corresponding band in the alkaline lysis ladder (containing 5'-OH termini), but migrates the same way as the cleavage product of a control RNase MRP digest (containing 5'-phosphate ends) as one would expect for a cleavage reaction catalyzed by RNase P holoenzyme and M1 RNA (Figure 2). Cleavage does not occur in the presence of other, non-catalytic RNAs that were transcribed and purified in the same way as M1 RNA; moreover, the cleavage efficiency increases with increasing amounts of M1 RNA used in the assay (data not shown). Again, we did not note cleavage of D-loop mtRNA by M1 RNA at the cleavage sites 1 and 3 of RNase MRP.

Cleavage does not occur in the absence of $MgCl_2$ (Figure 4) as expected for a reaction catalyzed by M1 RNA that requires $MgCl_2$ for activity and is only efficient at high $MgCl_2$ concentrations (Figure 4, 80–500 mM). No cleavage occurs at the RNase P RNA cleavage site in the presence of high $MgCl_2$ concentrations alone (Figure 4, mock, 100 mM $MgCl_2$).

These results show that *E.coli* RNase P cleaves mouse D-loop mtRNA exactly at an RNase MRP cleavage site.

DISCUSSION

Puromycin is known to inhibit protein synthesis by mimicking the amino-acylated 3'-end of a tRNA (22). In a hypothetical secondary structure model of M1 RNA a large domain of M1 RNA closely resembles the secondary structure of the tRNA binding center of 16S rRNA (23). So puromycin could interfer with tRNA binding by both RNase P and 16S rRNA. But as the secondary structure model of M1 RNA has been refined (24) the mode of inhibition of M1 RNA by puromycin remains unclear. Although we do not understand the mechanism of inhibition, sensitivity to puromycin seems to be a characteristic common to RNase P like enzymes. As the RNA components of these ribonucleases can be folded into similar structures (10, 11) and as they share a similar response to puromycin (Figure 1) one could hypothesize that also their mode of substrate recognition might be similar.

It is intriguing to find that both RNase MRP and RNase P cleave mouse D-loop mtRNA at a common site. It is possible that RNase MRP and RNase P share more commonalities for substrate recognition, than previously anticipated. Perhaps both RNase MRP and RNase P recognize their substrates by means of an external guide sequence (25), but we do not know what is (are) really the common structural element(s) required by each

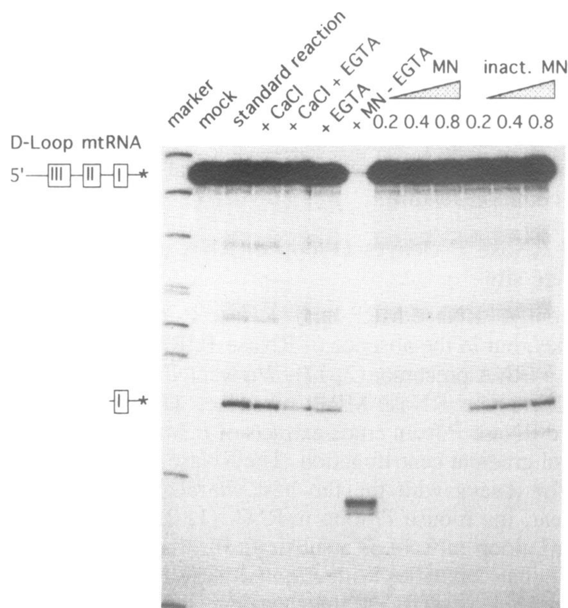

Figure 3. Processing of 3'-end labeled mouse D-loop mtRNA by *E.coli* RNase P is sensitive to pretreatment with micrococcal nuclease. 3'-end labeled D-loop mtRNA in standard reaction buffer R2 without enzyme was used as mock reaction. Micrococcal nuclease (MN) pretreatment and control reactions were carried out as described in Materials and Methods. Relevant reaction products are indicated as described in Figure 1.

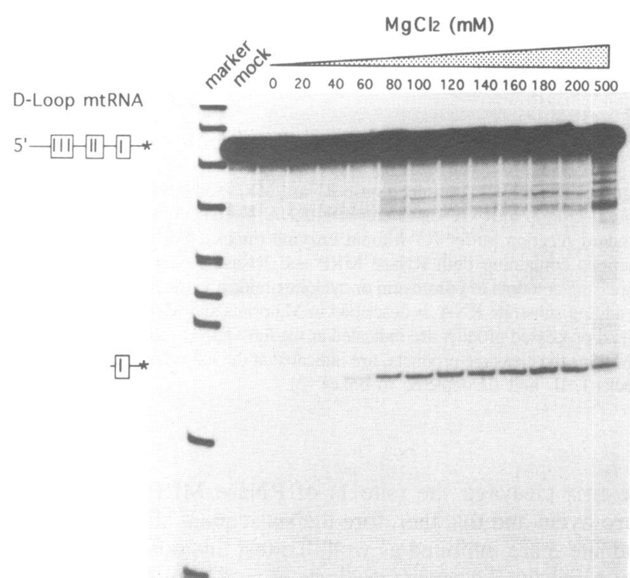

Figure 4. $MgCl_2$-dependent cleavage of mouse D-loop mtRNA by M1 RNA. 3'-end labeled mouse D-loop mtRNA in standard reaction buffer R1 (containing 50 mM Hepes-KOH, pH 7.6, 100 mM NH_4Cl, 100 mM $MgCl_2$) without (mock) or with 500 ng M1 RNA per assay. The other reactions were carried out in the same reaction buffer R1 with increasing concentrations of $MgCl_2$ (0–500 mM). M1 RNA dependent reaction products are indicated as described in Figure 1.

enzyme. Alternatively, recognition of the mouse D-loop mtRNA by RNase MRP and RNase P may occur by distinct modes but leading to the same cleavage product. Much is known about the secondary structural requirements of RNase P substrates but there is only little information about the possible secondary structures of D-loop mtRNAs (26). It is important to characterize the secondary structure of mouse D-loop mtRNA in order to understand the extent of possible commonalities for substrate recognition by RNase MRP and RNase P enzymes. Moreover, knowing the structural requirements for substrate recognition by RNase MRP may be useful in finding a nuclear substrate for RNase MRP that has been postulated to be a ribosomal RNA precursor (2, 5–7, 27) but has not been characterized yet.

There is little knowledge about mammalian mitochondrial transcript processing activities (28, 29) that are required for tRNA processing (30–32) and that could perhaps also participate in other mitochondrial RNA processing reactions (26, 33–35). Our results show that the mouse D-loop mtRNA is a mitochondrial model substrate for RNase MRP and RNase P enzymes to study the mechanisms of substrate recognition and catalysis. Since very few RNase MRP RNA copurifies with mitochondria (3), the partial overlapping specifities of *E.coli* RNase P and RNase MRP might reflect the evolutionary relatedness of an enzyme family rather than a conserved *in vivo* function.

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REFERENCES

1. Chang, D.D. and Clayton, D.A. (1987) *EMBO J.* **6**, 409–417.
2. Karwan, R., Bennett, J.L. and Clayton, D.A. (1991) *Genes Dev.* **5**, 1264–1276.
3. Kiss, T. and Filipowicz, W. (1992) *Cell* **10**, 11–16.
4. Topper, J. N., Bennett, J. L., and Clayton, D. A. (1992) *Cell* **70**, 16–20.
5. Kiss, T., Marshallsay, C. and Filipowicz, W. (1992) *EMBO J.* **11**, 3737–3746.
6. Yuan, Y., Singh, R. and Reddy, R. (1989) *J. Biol. Chem.* **264**, 14835–14839.
7. Schmitt, M.E. and Clayton, D.A. (1992) *Genes & Dev.* **6**, 1975–1985.
8. Altman, S. (1989) *Adv. Enzym.* **62**, 1–36.
9. Gold, H.A., Topper, J.N., Clayton, D.A. and Craft, J. (1989) *Science* **245**, 1377–1380.
10. Forster, A.C. and Altman, S. (1990) *Cell* **62**, 407–409.
11. Schmitt, M.E., Bennett, J.L., Dairaghi, D.J. and Clayton, D.A. *FASEB J.* **7**, 208–213.
12. Rossmanith, W. and Karwan, R. (1993), Mol. Biol. Rep., in press.
13. Karwan, R. (1993) *FEBS Lett.* **319**, 1–4.
14. Gold, H.A., Craft, J., Hardin, J.A., Bartkiewicz, M. and Altman, S. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5483–5487.
15. Bartkiewicz, M., Gold, H. and Altman, S. (1989) *Genes Dev.* **3**, 488–499.
16. Altman, S., Kirsebom, L. and Talbot, S. (1993) *FASEB J.* **7**, 7–14.
17. Bennett, J.L., Jeong-Yu, S. and Clayton, D.A. (1992) *J. Biol. Chem.* **267**, 21765–21772.
18. Doria, M., Carrara, G., Calandra, P. and Tocchini-Valentini, G.P. (1991) *Nucl. Acids Res.* **19**, 2315–2320.
19. Vioque, A. (1989) *FEBS Lett.* **246**, 137–139.
20. Maniatis, T. Fritsch, E.F. and Sambrook, J. (1989) Molecular Cloning, A Laboratory Manual. Cold Spring Harbor University Press, Cold Spring Harbour.
21. Reed, R.E., Baer, M.F., Guerrier-Takada, C., Donis-Keller, H. and Altman, S. (1982) *Cell* **30**, 627–636.
22. Robertson, H.D., Altman, S and Smith, J.D. (1972) *J. Biol. Chem.* **247**, 5243–5251.
23. Kuchino, Y. and Nishimura, S. (1989) In Dahlberg, J.E. and Abelson, J.N. *Meth. Enzymol.* **180**, 154–164.
22. Jiménez, A. (1976) *Trends Biochem. Sci.* **1**, 28–30.
23. Boehm, S. (1987) *FEBS Lett.* **220**, 283–287.
24. Haas, E.S., Morse, D.P., Brown, J.W., Schmidt, F.J. and Pace, N.R. (1991) *Science* **254**, 853–856.
25. Forster, A.C. and Altman, S. (1990) *Science* **249**, 783–786.
26. Brown, G.G., Gadaleta, G., Pepe, G., Saccone, C.and Sbisà, E. (1986) *J. Mol. Biol.* **192**, 503–511.
27. Reimer, G., Raska, I., Scheer, U. and Tan, E.M. (1988) *Exp. Cell Res.* **176**, 117–128.
28. Doersen, C.J., Guerrier-Takada,C., Altman,S. and Attardi, G. (1985) *J. Biol. Chem.* **260**, 5942–5949.
29. Manam, S. and van Tuyle, G.C. (1987) *J. Biol. Chem.* **262**, 10272–10279.
30. Ojala, D., Merkel, C., Gelfand, R.and Attardi, G. (1980) *Cell* **22**, 393–403.
31. Ojala, D., Montoya, J. and Attardi, G. (1981) *Nature* **290**, 470–474.
32. Sbisà, E., Tullo, A., Nardelli, M., Tanzariello, F. and Saccone, C. (1992) *FEBS-Lett.* **296**, 311–316.
33. Chang, D.D. and Clayton, D.A. (1985) *EMBO J.* **4**, 1559–1567.
34. Sbisà, E., Nardelli, M., Tanzariello, F., Tullo, A., and Saccone, C. (1990) *Curr. Genet.* **12**, 247–253.
35. Saccone C., Pesole, G., and Sbisà, E. (1991) *J. Mol. Evol.* **33**, 83–91.