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Conformation of yeast 18S rRNA. Direct chemical probing of the 5' domain in ribosomal subunits and in deproteinized RNA by reverse transcriptase mapping of dimethyl sulfate-accessible sites

Laurence Lempereur[1], Monique Nicoloso[1], Nadine Riehl[2], Chantal Ehresmann[2], Bernard Ehresmann[2] and Jean-Pierre Bachellerie[1]*

[1]Centre de Recherche de Biochimie et de Génétique Cellulaires du CNRS, Université Paul Sabatier, 118 route de Narbonne, 31062 Toulouse Cedex, and [2]Institut de Biologie Moléculaire et Cellulaire du CNRS, Laboratoire de Biochimie, 15 rue René Descartes, 67084 Strasbourg Cedex, France

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SUMMARY

The structure of the 5' domain of yeast 18S rRNA has been probed by dimethyl sulfate (DMS), either in "native" deproteinized molecules or in the 40S ribosomal subunits. DMS-reacted RNA has been used as a template for reverse transcription and a large number of reactive sites, corresponding to all types of bases have been mapped by a primer extension procedure, taking advantage of blocks in cDNA elongation immediately upstream from bases methylated at atom positions involved in the base-pair recognition of the template. Since the same atom positions are protected from DMS in base-paired nucleotides, the secondary structure status of each nucleotide can be directly assessed in this procedure, thus allowing to evaluate the potential contribution of proteins in modulating subunit rRNA conformation. While the DMS probing of deproteinized rRNA confirms a number of helical stems predicted by phylogenetic comparisons,it is remarkable that a few additional base-pairings, while proven by the comparative analysis, appear to require the presence of the bound ribosomal subunit proteins to be stabilized.

INTRODUCTION

Different kinds of evidence have accumulated during the last years which show that a wide range of functional activity may be performed by RNA molecules. In addition to their classical roles as protein-coding or "structural" molecules, some RNA chains or portions of RNAs (1, 2) do possess per se enzyme-like properties, which must closely depend upon a specific three-dimensional configuration of the RNA chain (3), thus making the precise mapping of secondary and tertiary structure features of utmost importance for a detailed understanding of RNA functions.

Although the comparative analysis of primary structure has proven to be a most potent approach for deriving secondary structure models for RNA (4-10) it also suffers from obvious limitations particularly on the dynamical aspects of RNA conformation. Moreover direct experimental informations are definitely required in order to identify conformation switches in RNA structure and to assess the structural effects of ligands.

A variety of nucleases and chemicals can be effective probes of RNA

structure, since their reactivity is highly dependent upon secondary and/or tertiary structure features (11-15). However, it has been difficult to use these probes onto long molecules, such as rRNAs, due to the lack of fast and resolutive mapping procedure. We therefore recently developed a new mapping method (16) in order to obviate this limitation. One of the key advantages of this method is that no prior end-labeling of RNA is required before reaction with the structural probes, which allows the probing of a native RNA structure, as in the form of a RNP complex. Another advantage is that information can be obtained at the sequence resolution level over the entire length of the RNA molecule, whatever its length. In our previous report, the reverse transcriptase mapping procedure had been applied to the case of an enzymatic structural probes (i.e. S1 nuclease). However, the bulky nucleases can only have access to a limited portion of the RNA configuration whereas a series of chemicals, such as DMS, can provide a wealth of detailed informations about the local conformation of individual nucleotides and the potential changes induced by various ligands (17). Accordingly in the present study we have applied the reverse transcriptase mapping approach to the case of a chemical structural probe, DMS. We have analyzed the 5'domain of yeast 18S rRNA (either as a naked "native" RNA molecule or as a part of the 40S ribosomal subunit), a region for which phylogenetic comparisons remain poorly informative due to the presence of some highly variable tracts. Our experimental data are discussed by reference to the previously proposed secondary structure models of small subunit rRNA (7, 18-21). During the preparation of this manuscript, an application of this method for analyzing the self-splicing intron of Tetrahymena rRNA has been reported (3).

MATERIALS AND METHODS

1. DNA Primers : The 320 5'terminal nucleotides of yeast 18S rRNA were probed by using 2 different DNA primers : the first one was a fragment of cloned mouse rDNA and the second one a synthetic oligonucleotide, which allowed us to analyze at sequence resolution segments 320 to 100 and 144 to 1 respectively (see Figure 3 for location).

a) Mouse primer : this heterologous primer was prepared for recombinant plasmid pMSE2, which contains the 5'part of mouse 18S rDNA (22). The 55 bp TaqI-TaqI fragment maps from position 370 to 425 along mouse 18S rDNA (i.e. positions 321 to 375 along yeast 18S rDNA (23)). Due to its location within a region which has been strongly conserved during the evolution of eucaryotes, the mouse primer can faithfully hybridize with yeast rRNA, particularly over

its 3'terminal nucleotides, thus allowing its elongation by reverse transcriptase. This mouse primer was obtained by direct TaqI digestion of total pMSE2 DNA followed by separation on a 6 % acrylamide non-denaturing gel. The purified double-stranded DNA fragment was 5' (^{32}P) end-labeled. A strand separation was carried out onto a 8 % acrylamide (Bis-acrylamide : acrylamide = 1/50) gel and the coding-strand was recovered for further analysis. All these steps were essentially performed according to the Maxam-Gilbert procedures (24).

b) Synthetic primer : The 28 mer primer was a synthetic oligonucleotide (a generous gift of Elf BioRecherches - Labège) coding for an evolutionary conserved sequence, position 147-174 from the 5'end of mouse 18S rRNA (22), which corresponds to yeast positions 145-172 (23).

2. Isolation of ribosomal subunits and rRNA : Saccharomyces cerevisiae wild type strain F1.100 : ATCC28383 (25) grown aerobically in 1 % yeast extract, 1 % bactopeptone, 1 % glucose, at 30°C, was harvested in middle logarithmic phase. Cells were washed twice in 10 mM Tris-HCl, pH 7.5, 5 mM Mg acetate, 10 mM KCl and 10 mM B-mercaptoethanol. Pelleted cells were ruptured by grinding with 1 vol. of .4 mm glass beads and 5 mM DTT was added to inhibit nuclease activity bound to 40S subunits (26). The cell homogenate was centrifuged at 10,000 g for 20 min, and ribosomes were collected by centrifugation at 105,000 g for 2 h. Ribosomal subunits were prepared by suspending the ribosomal pellet in the homogeneization buffer containing 0.5M KCl, and fractionated on a 10-30 % linear sucrose gradient in the same buffer (22,000 rpm, 16 h, SW28 rotor). Fractions containing the 40S subunits were pooled and precipitated with 0.65 volume ethanol at 0°C for 30 min. Ribosomal RNA was deproteinized by redissolution in 0.5 % SDS,10 mM Tris-HCl, pH 7.5, 100 mM LiCl, 1 mM EDTA, 5 mM dithiothreitol and 18S rRNA was recovered after centrifugation (26,000 rpm, 20 h., SW28 rotor) on a 5-25 % linear sucrose gradient in the same buffer.

3. RNA chemical modification : The general procedures were derived from Peattie (14).

a) Probing "native" rRNA and ribosomal subunit : 10 µg of 18S RNA or 20 µg of 40S subunit were preincubated in 200 µl of Na cacodylate 50 mM pH 7.0, 5 mM DTT, Mg Acetate 20 mM, NaCl 100 mM at 37° for 20 minutes and cooled on ice. Then, 0.5 µl of DMS (Janssen Chimica) was added. The solution was incubated for 10 or 15 min at 37°, then rapidly chilled and made 0.2M Na acetate pH 5.5 before precipitation with 3 volumes of ethanol. Ethanol precipitations, after dissolution of the sample in 0.3M Na acetate, were repeated twice in order to

ensure a thorough elimination of DMS. When the RNA structure had been probed within 40S ribosomal subunit, deproteinization of the DMS-reacted sample was necessary before mapping analysis : it was achieved by phenol extraction in the presence of 0.3 % SDS in 0.2M NaCl, 1 mM EDTA at room temperature, followed by ethanol precipitation.

b. Probing denatured RNA : 10 µg of 18S rRNA were dissolved in 300 µl of 50 mM sodium cacodylate, pH 7.0, 1 mM EDTA, 0.5 µl of DMS was added and the mixture was immediately brought at 90°C for 1 min., then rapidly chilled and processed as for native RNA. At this point the modified RNAs can be hybridized to the DNA primer.

4. Identification of methylated sites in RNA :

a. CDNA synthesis onto modified RNA templates : Conditions were essentially as reported for the mapping of nuclease accessible sites (16). The standard elongation mixture contained 5 µg (8 pmoles) of DMS-reacted 18S rRNA and 0.1-1 pmole of the labeled DNA primer in 30 µl. After the reaction was stopped by adding SDS/EDTA, RNA in the sample was hydrolyzed by alkaline treatment and cDNAs were analyzed onto 6 or 8 % acrylamide/8M urea sequencing gels (80 cm x 23 cm x 0.04 cm) as in (16).

b. Analysis at sequence resolution : The spectrum of labeled cDNA obtained onto DMS-reacted RNA was sized at sequence resolution by analyzing the samples in parallel to a set of base-specific elongation reactions (27) carried out on control unmodified 18S rRNA using the same DNA primer, as in (16).

RESULTS

Mapping of DMS modifications : When the oligonucleotide primer was hybridized to DMS-reacted yeast 18S rRNA and elongated with reverse transcriptase, a complex spectrum of prematurely terminated cDNAs was obtained instead of the fully elongated chains which were normally synthesized on an unreacted template (Fig. 1). These chain terminations resulted from methylations of the template at base positions involved in Watson-Crick base-pairing (see Discussion) : accordingly each band of prematurely terminated cDNA was indicative of a blocking modification onto the next upstream nucleotide position in the band pattern of the RNA template sequence.

As expected from the known dependence of DMS reactivity upon RNA conformation, the pattern of reverse-transcriptase-blocking modifications was clearly affected by changes in 18S rRNA structure : when 18S rRNA was reacted with DMS after heat-denaturation instead of "native" conditions, an array of additional cDNA bands was observed (Fig. 1). When the DMS treatment was per-

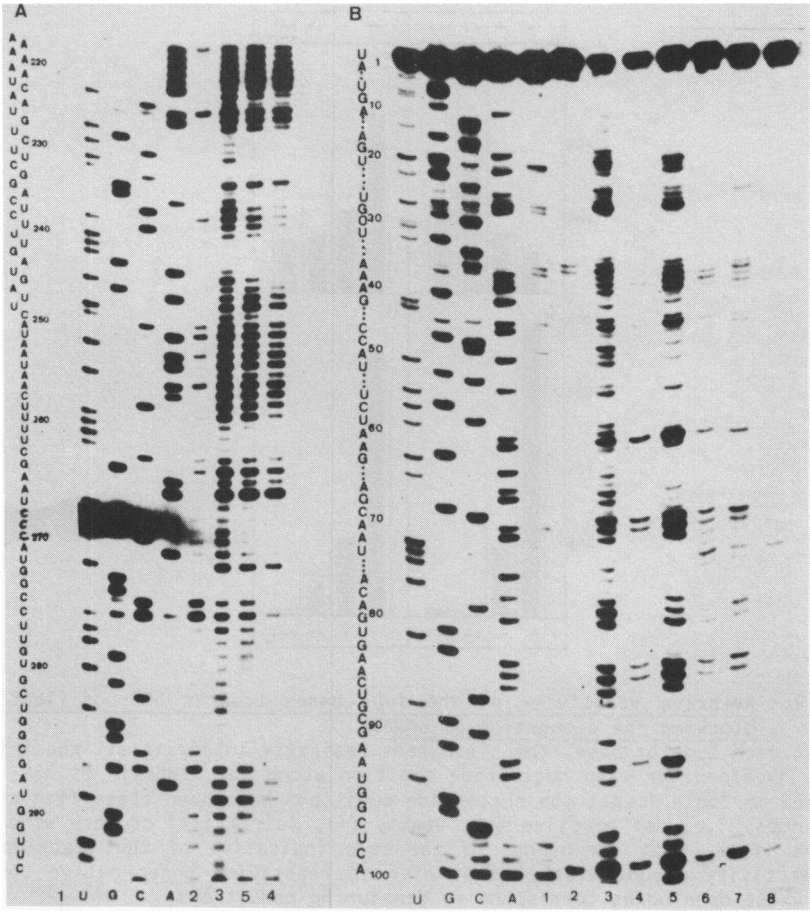


Figure 1 : cDNA products generated onto DMS-reacted 18S rRNA

A) DMS reaction was carried out on deproteinized RNA, either in denaturing conditions (lane 3) or in native conditions (lanes 4 and 5, 10 min. and 15 min. reactions respectively), and the reverse transcriptase elongation products of the mouse primer were analyzed onto a 6 % polyacrylamide/8M urea gel, in parallel with a set of the four dideoxynucleotide sequencing reactions (lanes U, G, C, A). As controls, unmodified rRNAs were also analyzed by primer elongation in the presence of the four normal NTPs, either directly (lane 1) or after heat-denaturation (1 min. at 90°C, lane 2).

B) Elongation products of the synthetic 28-mer primer analyzed onto a 8 % polyacrylamide/8M urea gel. RNA samples were identical to the ones analyzed in A (with same lane numbers in both gels), except for the additional analysis of RNA samples probed in the native ribosomal subunit (lanes 6 and 7, corresponding to 10 min. and 15 min. DMS-reaction respectively). Lane 8 shows a control elongation performed on unreacted rRNA purified from isolated ribosomal subunits.

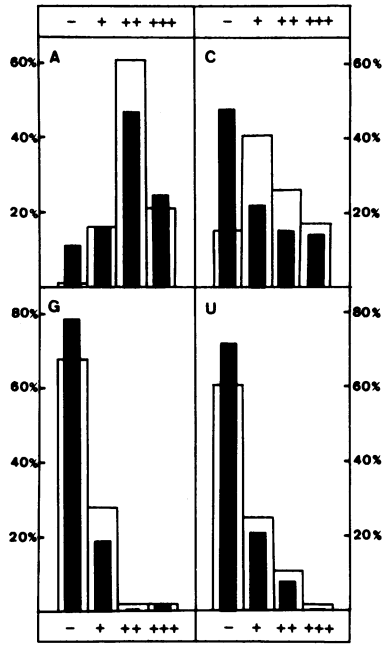


Figure 2 : Relative reactivity of the four bases towards DMS-modifications blocking the elongation of cDNA.

For each type of base, the histograms basically integrate all the informations obtained for each nucleotide position along the 5'domain of 18S rRNA as listed in Table 1. All the nucleotide positions have been classified among four groups, i.e. not reactive (-), weakly (+), fairly (++) or very strongly reactive (+++), with the height of the bars indicative of the fraction in this reactivity group. Black boxes refer to reactivity under native conditions, while open boxes correspond to denaturing conditions.

formed onto 40S ribosomal subunits, instead of deproteinized rRNA, the spectrum of cDNA bands was reproducibly altered at a number of specific positions.

As inferred from the relative intensities of cDNA bands terminated at the nucleotide position immediately upstream, the accessibility of each base of the 5'domain of yeast 18S rRNA was determined in a semi-quantitative way for deproteinized rRNA (either in native or in denatured form) and for rRNA in the ribosomal subunit RNP. The results (listed in Table 1) were examined in terms of secondary structure of the 18S rRNA, after potential correction for the presence of endogenous nicks in the template (control columns in Table 1). Their significance was also assessed by systematical reference with both the reactivity of vicinal nucleotides and with the average "intrinsic"

Table 1 : Reactivity of individual nucleotides of yeast 18S rRNA toward DMS modifications blocking reverse transcription in denatured or native "naked" RNA and in native 40S subunit. Positions are classified as very strongly (+++), fairly (++), weakly (+) or barely (+/-) reactive or unreactive (-). Unreacted rRNAs were also processed for control elongation, with + signs in the column denoting significant levels of endogenous nicks on the phosphodiester bond 3' to the numbered base.

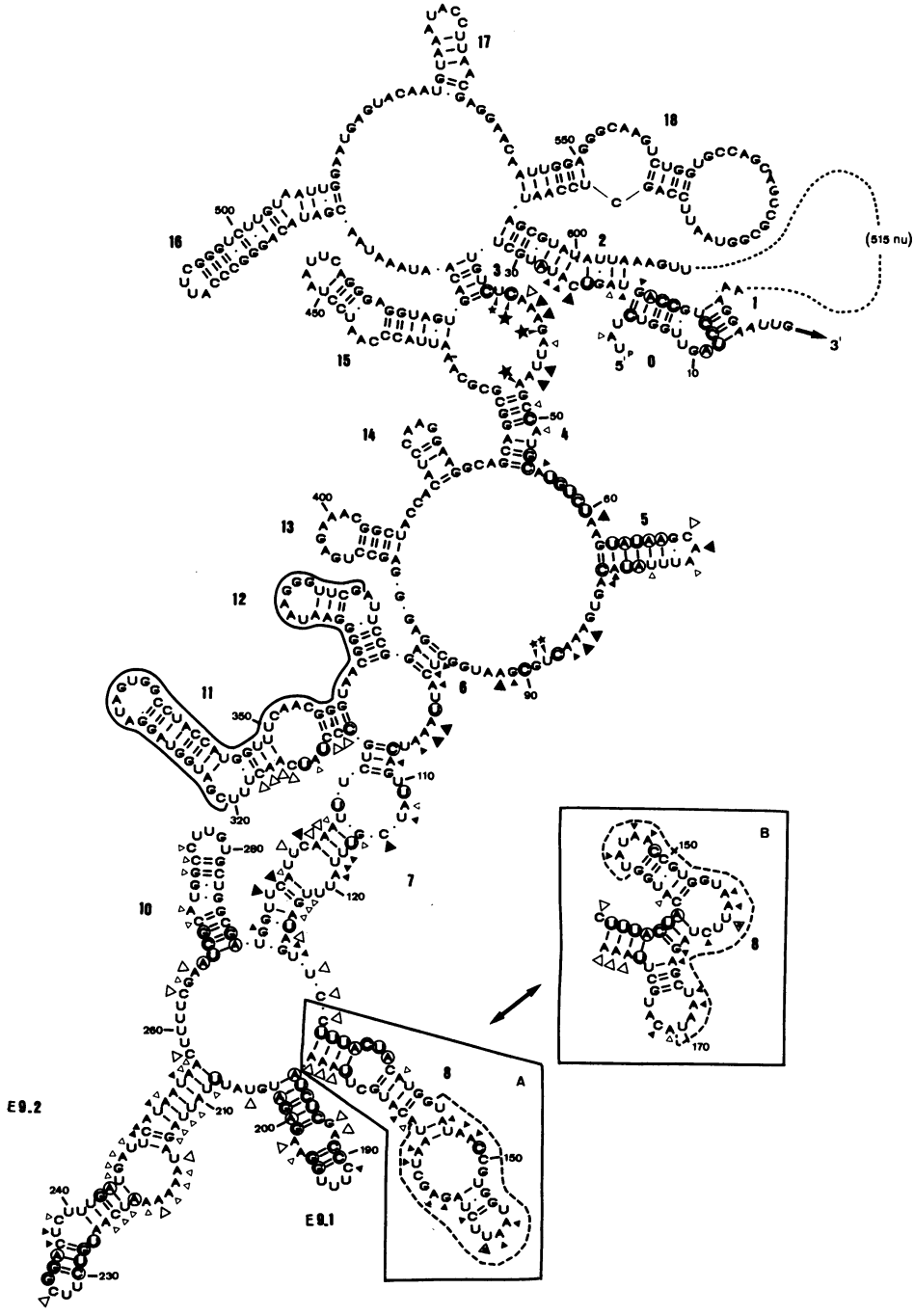
	Control			DMS-reacted				Control			DMS-reacted		
	den. RNA	nat. RNA	nat. s.u.	den. RNA	nat. RNA	nat. s.u.		den. RNA	nat. RNA	nat. s.u.	den. RNA	nat. RNA	nat. s.u.
C317	+/-	-	-	+++	+++	-	U267	-	-	-	+	+/-	-
A316	-	-	-	+++	+++	-	A266	-	-	-	+	-	-
A315	-	-	-	+++	+++	-	A265	-	-	-	+++	+++	-
C314	+	+/-	+/-	+++	+++	+/-	G264	+/-	-	-	+	+	-
U313	-	-	-	+	-	-	C263	-	-	-	++	++	-
A312	-	-	-	++	++	-	U262	-	-	-	+/-	+/-	-
U311	-	-	-	++	+	-	U261	-	-	-	+/-	+/-	-
C310	-	-	-	++	++	-	U260	-	-	-	+/-	+/-	-
C309	++	+	-	+++	+++	-	U259	-	-	-	+/-	+/-	-
C308	-	-	-	+	-	-	C258	-	-	-	++	++	-
G307	-	-	-	-	-	-	A257	-	-	-	++	++	+/-
U306	-	-	-	-	-	-	A256	-	-	-	++	++	+/-
C305	-	-	-	-	-	-	U255	+	+/-	-	+	+	+
U304	-	-	-	+/-	+	-	A254	-	-	-	+	+	+
U303	-	-	-	+/-	-	-	A253	-	-	-	++	++	+
U302	-	-	-	-	-	-	U252	+	-	-	+	+	+
A301	-	-	-	++	++	-	A251	-	-	-	+	+	+
A300	-	-	+/-	+++	+++	++	C250	+	-	+/-	+	+	+
A299	-	-	+/-	+++	+++	++	U249	+	-	+/-	+	+	+
C298	++	+	+	+++	+++	+++	U248	-	-	-	+	+	-
U297	-	-	-	++	++	-	A247	-	-	-	++	++	-
U296	-	-	-	-	-	-	G246	-	-	-	+	+	-
A295	-	-	-	++	++	+	U245	-	-	-	+	+	-
C294	+	-	+	+++	+++	+++	A244	-	-	-	++	+/-	-
U293	-	-	-	++	++	++	G243	-	-	-	+/-	-	-
U292	-	-	-	-	-	-	U242	-	-	-	+/-	+/-	-
G291	-	-	-	+/-	+/-	+/-	U241	-	-	-	+/-	+/-	-
G290	+	-	+/-	+	+	+	U240	-	-	-	+/-	+/-	-
U289	+	-	++	+/-	+/-	+/-	C239	-	-	-	+	+	-
A288	-	-	+/-	+++	+	+	U238	-	-	-	+	+	+
G287	-	-	+/-	++	+/-	+	C237	+	-	-	+	+	+
C286	++	-	-	+/-	+/-	+/-	A236	-	-	-	++	+	+
G285	+	-	-	+/-	+/-	+/-	G235	+	-	-	+	-	-
G284	-	-	++	+/-	+/-	-	G234	+	-	-	+/-	-	-
U283	-	-	+++	+/-	+/-	-	C233	-	-	-	++	++	+
C282	-	-	+++	+/-	+/-	-	U232	-	-	-	+/-	+/-	-
G281	-	-	+++	+/-	+/-	-	U231	-	-	-	-	-	-
U280	-	-	++	+/-	+/-	-	C230	-	-	-	+	-	-
G279	-	-	++	+/-	+/-	-	U229	-	-	-	+	-	-
U278	-	-	+++	+/-	+/-	-	G228	-	-	-	+	-	-
U277	-	-	++	+/-	+/-	-	U227	-	-	-	+/-	-	-
C276	-	-	+	+	+	-	A226	-	-	-	+	+	-
C275	++	+	-	+	+	-	A225	-	-	-	++	++	-
G274	+	-	-	+	+	-	C224	+	+	+/-	+	+	-
G273	+/-	-	-	-	-	-	U223	-	-	-	+	+	-
U272	-	-	-	-	-	-	A222	-	-	-	++	+	-
A271	-	-	-	++	++	+	A221	-	-	-	++	++	-
C270	-	-	-	+	+	+/-	A220	-	-	-	+++	+++	-
G269	-	-	-	+	-	+/-	A219	-	-	-	++	++	-
C268	-	-	-	+	+/-	-	A218	-	-	-	++	++	-

	Control			DMS-reacted				Control			DMS-reacted		
	den. RNA	nat. RNA	nat. s.u.	den. RNA	nat. RNA	nat. s.u.		den. RNA	nat. RNA	nat. s.u.	den. RNA	nat. RNA	nat. s.u.
A217	-	-	-	++	++	-	A162	-	-	nd	-	-	nd
U216	+	+	-	++	++	-	U161	-	-	nd	-	-	nd
A215	+	-	-	+	+	-	C160	-	-	nd	+	+	nd
G214	+	-	-	+	+	-	U159	-	-	nd	-	-	nd
A213	+	-	-	++	++	+/-	U158	-	-	nd	++	++	nd
U212	-	-	-	+	+	+/-	A157	-	-	nd	++	++	nd
U211	-	-	-	+	+	-	A156	-	-	nd	++	++	nd
A210	+	-	-	+	+	-	U155	+	+	nd	+/-	+/-	nd
U209	-	-	+	+	+	-	G154	-	-	nd	-	-	nd
U208	-	-	-	+	+/-	-	G153	-	-	nd	-	-	nd
U207	-	-	-	+/-	+/-	-	U152	-	-	nd	-	-	nd
A206	++	++	-	++	++	-	G151	-	-	nd	-	-	nd
U205	-	-	+	++	++	-	C150	-	-	nd	-	-	nd
G204	+	-	-	+/-	+/-	-	C149	-	-	nd	++	-	nd
U203	-	-	-	+/-	+/-	-	A148	-	-	nd	++	++	nd
A202	-	-	-	+	-	-	A147	-	-	nd	++	++	nd
G201	-	-	-	+/-	-	-	U146	-	-	nd	-	-	nd
A200	-	-	-	++	-	-	A145	-	-	nd	++	++	nd
G199	-	-	-	+/-	-	-	U144	-	-	nd	-	-	nd
A198	-	-	-	+++	+++	++	G143	-	-	-	-	-	-
A197	-	-	-	++	++	-	G142	-	-	-	-	-	-
G196	+	-	+	+	-	-	U141	-	-	-	-	-	-
G195	+	-	-	+	-	-	A140	+	+	-	++	++	-
U194	-	-	-	-	-	-	C139	+++	+++	+++	-	-	-
U193	-	-	-	-	-	-	A138	+	+	+	+++	+	+
U192	-	-	-	-	-	-	U137	+	+	+	+++	+	+
C191	-	-	+++	+	+	+	C136	-	-	-	+	-	+/-
C190	-	-	++	+	-	-	A135	-	-	-	+++	++	+
C189	-	-	-	+	-	-	U134	-	-	+	+++	+	+
A188	-	-	-	+++	+++	++	U133	-	-	-	+	-	-
G187	-	-	-	+++	+++	-	U132	-	-	-	+	-	-
C186	-	-	-	+	-	-	C131	+/-	+/-	+/-	+++	+++	+
U185	-	-	-	+	-	-	C130	+/-	+/-	+/-	+++	+++	++
C184	-	-	-	+	-	-	U129	-	-	-	++	++	+
U183	-	-	-	+	-	-	U128	-	-	-	+	+	+
A182	-	-	-	+++	+	-	G127	-	-	-	+	+	+
A181	-	-	-	+++	+++	-	A126	-	-	-	+++	+++	++
A180	-	-	-	+++	+++	+	U125	-	-	+	++	-	+
A179	-	-	-	+++	+++	+	A124	-	-	-	++	++	+
U178	+++	+++	+++	+/-	+/-	+	G123	-	-	-	+	+	+/-
U177	-	-	-	+/-	+/-	-	U122	-	-	-	+	+	+/-
C176	-	-	-	+/-	+/-	-	U121	-	-	-	+/-	+/-	+/-
G175	+	-	-	+/-	+/-	-	U120	-	-	-	-	-	-
U174	-	-	-	+/-	+/-	-	A119	-	-	-	++	++	++
A173	-	-	-	+	+	-	U118	+	+	+	+	+	+
C172	+	+	+	+	+	+	U117	+/-	+/-	+	+	+	+
A171	-	-	-	++	++	+/-	U116	+/-	+/-	+/-	+	-	-
U170	-	-	nd	-	-	nd	G115	+/-	+/-	+/-	+	+	-
A169	-	-	nd	+	+	nd	C114	-	-	-	++	++	++
A168	-	-	nd	++	++	nd	U113	-	-	-	+	+	+
U167	-	-	nd	+	+	nd	A112	-	-	-	++	++	+
C166	-	-	nd	-	-	nd	U111	-	-	++	++	+	+/-
G165	+	-	nd	-	-	nd	U110	-	-	-	+/-	+/-	+/-
A164	-	-	nd	++	++	nd	G109	-	-	-	+/-	+/-	+/-
G163	-	-	nd	+	+	nd	A108	-	-	-	++	++	++
C107	+	+	++	+++	++	+	G53	-	-	-	+	-	+/-
U106	-	-	-	-	-	-	U52	-	-	-	-	-	+
A105	-	-	-	+	+	+	A51	-	-	-	++	++	+
A104	-	-	-	+++	+++	+++	C50	-	-	-	++	-	-
A103	-	-	-	+++	+++	+++	C49	+	+	+	++	++	-
U102	+	+	++	++	+/-	+	G48	-	-	-	-	-	-

	Control			DMS-reacted				Control			DMS-reacted		
	den. RNA	nat. RNA	nat. s.u.	den. RNA	nat. RNA	nat. s.u.		den. RNA	nat. RNA	nat. s.u.	den. RNA	nat. RNA	nat. s.u.
U101	-	-	-	-	-	-	A47	-	-	-	+	+	+++
A100	-	-	-	++	++	++	A46	+	+	+	++	+++	++
C99	++	++	++	+	+	+	U45	-	-	-	++	++	++
U98	+	+	+	+	+	+	U44	-	-	-	-	-	-
C97	+	+	+	+	+	+	A43	-	-	-	++	++	+
G96	-	-	-	-	-	-	G42	+/-	+/-	+/-	+/-	+/-	++
G95	-	-	-	-	-	-	A41	+/-	+/-	-	++	+++	+++
U94	-	-	-	-	-	-	A40	+/-	+/-	-	++	+++	+++
A93	-	-	-	+	+	-	A39	+/-	+/-	+	++	+++	++
A92	-	-	-	++	+++	+++	C38	+	+	+	+	+/-	++
G91	-	-	-	+	+	+	U37	-	-	-	+/-	+/-	+
C90	-	-	-	++	+/-	+	C36	-	-	-	++	+	-
G89	-	-	-	-	-	+	U35	-	-	-	-	-	-
U88	-	-	-	-	-	+/-	G34	-	-	-	+/-	+/-	-
C87	-	-	-	+	-	+/-	U33	-	-	-	-	-	-
A86	-	-	-	++	++	++	U32	-	-	-	-	-	-
A85	-	-	-	++	+++	+++	C31	-	-	-	-	-	-
A84	-	-	-	++	+++	+++	G30	-	-	-	-	-	-
G83	-	-	-	-	-	-	U29	-	-	-	-	-	-
U82	-	-	-	-	-	-	A28	++	++	+	+++	++	++
G81	-	-	-	-	-	-	U27	++	++	+	+	+	+
A80	-	-	-	++	++	++	A26	+	+	-	++	++	++
C79	+	+	+	+++	++	++	C25	+	+	+	++	++	++
A78	-	-	-	++	++	++	U24	-	-	-	+/-	-	-
U77	-	-	+	+	-	+	G23	-	-	-	-	-	-
A76	-	-	+	+	+/-	-	A22	-	-	-	++	++	+
U75	-	-	+++	+	+	-	U21	+	+	+	+	+	+
U74	-	-	+++	-	-	-	G20	+	+	+	+	+	+
U73	-	-	++	-	-	-	A19	-	-	-	++	+/-	-
A72	-	-	++	++	++	-	C18	-	-	-	++	+/-	+/-
A71	-	-	-	++	+++	+++	C17	-	-	-	+	-	-
C70	+	+	++	+++	+++	++	G16	-	-	-	-	-	-
G69	-	-	-	+/-	+/-	-	U15	-	-	-	-	-	-
A68	-	-	-	++	+/-	-	C14	-	-	-	++	+/-	-
A67	-	-	-	++	+	+	C13	-	-	-	+/-	-	-
U66	-	-	-	+/-	-	-	U12	-	-	-	+/-	-	-
A65	-	-	-	+	+/-	-	A11	-	-	-	++	+/-	-
U64	-	-	-	+/-	-	-	G10	-	-	-	-	-	-
G63	-	-	-	-	-	-	U9	-	-	-	-	-	-
A62	-	-	-	+	+	-	U8	-	-	-	-	-	-
A61	-	-	-	+++	+++	+++	G7	-	-	-	-	-	-
U60	-	-	-	++	+	+/-	G6	-	-	-	-	-	-
C59	-	-	-	++	-	+/-	U5	-	-	-	-	-	-
U58	-	-	-	+/-	-	-	C4	-	-	-	++	-	-
G57	-	-	-	+/-	-	-	U3	-	-	-	-	-	-
U56	-	-	-	+/-	-	-	A2	-	-	-	++	++	-
A55	-	-	-	++	++	++	U1	-	-	-	-	-	-
C54	-	-	-	+	-	+/-							

reactivity observed for the corresponding base (see below).

Base reactivity : While adenine proved to be the preferential target for DMS-induced elongation blocks, as recently reported by others (3), the three other bases also reacted to significant extents. A detailed account of the relative base reactivities observed for the 5' domain of yeast 18S rRNA is given in Fig. 2 which summarizes the semi-quantitative informations of Table 1.


(515 nu)
5'
3'
E9.2
E9.1
A
B

It is noteworthy that even in conditions (90°C, low salt concentration) which should minimize secondary structures, significant differences were reproducibly observed in the reactivity level of a given base, depending upon its position along the sequence, which could be indicative either of the persistence of some base-paired interactions or of a peculiar sensitivity of the probe to unknown features of local conformation. In heat-denatured 18S rRNA, almost 100 % of the As which have been analyzed were reactive to some extent, as compared to corresponding values of 85 % for Cs, 40 % for Us and only 30 % for Gs. The same order of base reactivity was still observed when only strong or fair reactivity levels were taken into account : 80 % of As were strongly or fairly reactive, as compared to corresponding values of 43 %, 15 % and 5 % for Cs, Us and Gs respectively.

DMS accessibility and secondary structure of 18S rRNA : The most clear-cut informations directly relevant to the higher order organization of the small subunit rRNA are summarized in Fig. 3, on a secondary structure consensus model. These data fall into several classes. The most simple case corresponds to the positions which displayed a marked decrease in DMS reactivity in "native" conditions as compared to denatured RNA, which can be most readily interpreted as indicative of an involvement in a Watson-Crick base-paired interaction in native rRNA. These nucleotides (circled in Fig. 2) are of two sorts : on one hand, for a number of helical stems of the model, there is a close correlation between the base-pairing predictions and the observed protections in native RNA : this is particularly striking for helices 0, 4, 5, 9.1 and for parts of helices 9.2 and 10. On the other hand, for a sizable fraction of these circled nucleotides, the observed protection is not correlated to any proposed secondary structure, as observed particularly for a

Figure 3 : DMS modifications blocking the elongation of cDNA mapped within the secondary structure of yeast 18S rRNA.

Whereas more than 600 of the 5'most nucleotides are represented, DMS probing data were restricted to region 1-320 (with the tract hybridizing to the mouse rDNA primer denoted by an overline). Helices have been numbered as proposed by Nelles et al. (21), except for the presence of additional helix 0. An alternative folding for helix 8 is shown in the inset.

Nucleotides accessible to DMS modification in denatured RNA but protected in "native" RNA are circled. Black arrow-heads points to nucleotides which remain reactive both in "native" RNA and in ribosomal subunits (with the size of the arrow-head indicative of the level of reactivity). Hollow arrow-heads denote nucleotides which are reactive in "native" RNA but are protected in the ribosomal subunits. The few nucleotides which are more accessible in the RNP subunit than in naked "native" RNA are denoted by stars. The dotted overline depicts a region for which structure-probing data for 40S subunits were not available.

pentanucleotide tract starting at position 56.

This also could be the case for the protected heptanucleotide starting at position 132, since it is noteworthy that its involvement in a 4 base-pair interaction - so as to extend helix 8 as compared to previous models - can only be considered as tentative as this stage : it is definitely not supported by comparative analysis (21) and the results of DMS probings do not favour unequivocally this possibility, since positions on the opposite strand remain readily reactive in native RNA (the 3 As at 179-181).

In addition to these unpredicted protections, another kind of unexpected result was obtained. A large fraction of the nucleotides which are base-paired in the proposed model did not display the differential protection in native RNA as compared to denatured RNA : they remained readily susceptible to DMS-induced elongation blocks whatever the probing conditions : this is most dramatic for helix 7 and for parts of helices 9.2 and 10. It is remarkable that these stem regions appear rather unstable as predicted on a thermodynamical basis (28) in the absence of bound subunit proteins. As for helix 8, it is noteworthy that whereas the alternative folding proposed in the inset (Fig. 3) much better accomodates our experimental datas, it is clearly not supported by comparative evidence.

Influence of bound ribosomal subunit proteins on DMS reactivity : The spectrum of nucleotide accessibility was not drastically modified when DMS probing was carried out on the ribosomal subunit : a large number of positions remained reactive to a similar extent (black arrow-heads in Fig. 3). However a number of very significant changes were reproducibly detected which deserve comment.

Firstly, some nucleotides which were easily accessible in "native" deproteinized RNA were protected from DMS modification in the ribosomal subunit (denoted by hollow arrow-heads in Fig. 3). Several of these differential protections do correspond to nucleotides which are either located in single-strand segments (see for example nucleotides 129-131, 216-221 or interior loop of helix 9.1) or at bulged positions (helices 4 and 10). However a fractions of them also map at positions which were predicted to be paired in the model, thus raising the possibility that subunit proteins may contribute in some way in stabilizing some stem structures which, although supported by phylogenetic arguments, were found to be easily accessible to the probe in "native" deproteinized RNA : this is the case for the loop-proximal region of helix 10 or the loop-distal region of helices 9.2 and 11.

Secondly, for a few nucleotides, the opposite effect was observed, i.e.

reactivity was significantly enhanced in the ribosomal subunit as compared to "native" deproteinized RNA (and even as to denatured deproteinized RNA) : this is most dramatic for 3 nucleotides which are very close from each other on a single-strand tract linking helices 3 and 4.

DISCUSSION

I. The method

a. General requirements for the structure probing experiment : Any degradation of the RNA template will result in the generation of unwanted cDNA bands, superimposed to the probe-specific pattern, which can obscure the interpretation of the data at some nucleotide positions. A particular care must be taken in order to preserve the integrity of the rRNA sample before and after submitting it to the action of the structure-specific reagent. Accordingly a control elongation reaction performed on unmodified rRNA is always required in order to unambiguously assess the actual effect of the chemical modification at each nucleotide position. It is noteworthy that a few positions along yeast 18S rRNA sequence appeared more particularly sensitive to endogenous nucleolytic cleavages taking place during purification, particularly during the handling of 40S ribosomal subunit.

As for the extent of chemical reaction, it should be kept ideally below the level of one nucleotide modification per RNA molecule, since the additional "hits" cannot be expected to represent reliable indexes of native structure, due to potential conformation changes following the first modification. It must be stressed also that low reaction levels are also a prerequisite of this mapping procedure itself : if multiple modifications are located upstream from the primer on the same template molecule, only the primer-proximal modified site can be identified by reverse transcription. The extent of chemical modification was easily monitored by assessing the proportion of full-size cDNAs among the entire population of elongated primers.

b. Reactivity of the chemical probe : All nucleic acid bases are susceptible to DMS alkylation in neutral aqueous solutions, however large differences are observed in the reactivity of each ribonucleotide (29) with puric bases more susceptible to methylation (with G > A) than pyrimidic bases (with C > U). At a neutral pH, the reaction is highly selective for a unique position within each base : DMS methylates preferentially the N-7 of G, N-1 of A, N-3 of C and U. Since the reaction can only take place if these groups are not involved in structural interactions in the RNA, the DMS reactivity of individual nucleotide may provide direct informations on the higher-order organization

of the RNA molecule. Potentially reactive positions in A, C and U are involved in Watson-Crick base-pairings in an RNA A helix, thereby allowing DMS-probing of the secondary structure status of these nucleotides. On the contrary the guanosine methylation can only provide information on tertiary interactions, since the N-7 position is not involved in base-pair H-bondings. c) The mapping of modified nucleotides : The weakening of the glycosylic bond between the alkylated base and the ribose moiety of the nucleotide can be ultimately used to generate a chemical scission of the phosphodiester chain. Sites of these subsequent nucleolytic breakages could then be precisely mapped by reverse transcriptase elongation of a DNA primer, as reported previously in the case of enzymatic probes (16). In fact, one may predict that the chemical cleavage step is not required for mapping most DMS modifications : since methylation of A, C and U can only take place on atoms potentially involved in Watson-Crick base-pairing, these DMS-modified bases can no longer serve as a template for reverse transcription, thereby resulting in elongation blocks (cDNAs ending one nucleotide position before any modified A, C or U residues along the template). As for the N-7 methylation of G, one may expect that the reverse transcriptase is "blind" to this modification since it does not alter the base-pairing potential of this nucleotide : direct evidence has already been reported, using E. coli 16S rRNA as a template (30), that the reverse transcription is not stopped (nor paused) at m^7G, as opposed to m^2G. Our results are in full agreement with this prediction : only a very slight fraction of cDNA elongation blocks do map immediately before G residues (Figure 2), although this nucleotide is the most reactive toward DMS. Accordingly the N-7 methylation of G can only be detected by the reverse transcriptase approach after site-specific cleavage of the modified template which can be achieved by sodium borohydride and aniline treatment (31) : in these conditions, a large proportion of elongation blocks then map immediately before G residues (not shown). As for the marginal fraction of elongation stops observed immediately before G when reverse transcription is directly carried out on an uncleaved template (Figure 2), they most likely correspond to N-1 modification, a minor reaction consecutive to a prior N-7 modification (29) which thus provides a probe for the secondary structure status of G residues.

Another mapping approach has been recently proposed for identifying sites accessible to chemical probes at sequence resolution in large RNAs, which also obviates the need of a labeling of RNA prior to structure probing (32). However since that technique involved the generation of specifically

tailored RNA fragments and their subsequent end-labeling and aniline-induced cleavages, it definitely appears less straightforward, more difficult to carry out on a routine basis and finally potentially less informative than the present reverse transcription approach.

d. Contribution of the DMS data to the assessment of the secondary structure model : In the present study we have analyzed a domain of eucaryotic 18S rRNA for which a secondary structure folding is most difficult to predict on the sole basis of comparative sequence analysis, particularly due to the presence of a cluster of tracts which are highly variable in the evolution (these three tracts, which have no clear procaryotic counterparts and which are generally enlarged in higher eucaryotes (21, 20), map over helical features 8, E 9.1 and E 9.2). Accordingly, it is not surprising that most of the major differences between published secondary structure models for eucaryotic small subunit rRNA (19-21, 33-35) involve the tracts engaged in the above mentioned stems, while all the other helices, numbered from 1 to 11, may be more or less considered as consensus proposals (except for helix 7, not proposed in a Xenopus model (34)). Consequently, the derivation of direct experimental evidence by using structural probes may represent a key step in testing the folding models, solving their discrepancies and assessing the contribution of the subunit protein moiety in the secondary structure organization of the rRNA molecule. The present results on DMS accessibility can be most easily interpreted as favouring the existence of several of the proposed helices in deproteinized RNA, in native conditions. This is plainly apparent for helices 0 and 2, for which comparative evidence is extensive. This is also clear for helices 4 and 5, which were proposed in most eucaryotic models despite a lack of comparative proofs (eucaryotic helix 5 does not represent a direct equivalent of its procaryotic counterpart and its potential stem may be severely altered in various eucaryotes). This is even more obvious in the case of the eucaryote-specific helix E 9.1, which remains poorly tested on a comparative basis. On the other hand, the DMS reactivity in "native" rRNA of nucleotides which could have been expected to be protected through predicted base-pairing may indicate that these base-pairings are quite unstable in the absence of the subunit proteins (examples of such a situation may be found in parts of helices 9.2, 10 and 11). Of course these observations do not contradict the conclusions obtained by the comparative approach since the occurrence of compensatory base changes in the evolution is merely indicative of base-pairings taking place in the subunit structure (and not in deproteinized RNA). Moreover it is noteworthy that these concerted base changes do not even

prove that the pairings are permanent but solely that they may be required at some specific stage of the ribosome cycle. Accordingly such unstable stems may be directly relevant to the dynamics of ribosome structure, by providing the flexible elements involved in biologically significant conformational switches of the subunit rRNA.

The case of nucleotides which are not predicted to be paired by the secondary structure model(s) but are nevertheless differentially protected from DMS in "native" rRNA (as compared to denatured rRNA) also appears rather intriguing. However these results could be most likely explained by the presence of tertiary interactions between more or less distant segments of the RNA molecule which do not participate in secondary structure pairings. In this hypothesis, it is noteworthy that a candidate for interacting with the unexpectedly protected pentanucleotide (positions 56-60) located between helices 4 and 5 can be found in the loop of helix 13 (positions 397-401). Obviously more direct informations are needed in order to test this possibility, such as the ones that can be derived from cross-linking studies (36).

While the pattern of DMS reactivity on 18S rRNA reveals a number of dramatic changes in the presence of bound subunit proteins, these data cannot provide an unequivocal answer about the contribution of the protein moiety to the secondary structure organization of the rRNA molecule. A first type of effects corresponds to a decreased reactivity of some nucleotides in the subunit RNP as compared to "native" deproteinized rRNA. Such results can in fact be interpreted both ways : the first possibility is that these nucleotides do not participate in Watson-Crick base-pairings, neither in deproteinized RNA nor in the native subunit, but are merely shielded from the chemical probe in the subunit by a close interaction with a ribosomal protein. Alternatively, the protection in the subunit could indicate the formation (or stabilization) of a base-paired interaction consecutive to the presence of the ribosomal proteins. In some cases, the presence of compensatory base changes among the eucaryotic sequences at these differentially protected positions may help to solve this ambiguity then strongly favouring the second possibility, as discussed above for portions of helices 9.2 (the loop distal stem) and 10 (for its loop-proximal half). The other type of effect detected at a few nucleotide positions, corresponding to an enhanced reactivity in the subunit, is not straightforward to interpret either : it must be stressed that the enhanced reactivity cannot be simply taken to indicate that stem-structures, present in "native" deproteinized RNA and involving these few nucleotides (such as positions 38, 42 and 47) were melted out in the RNP

subunit, as a result of protein contribution. Instead, they could be merely indicative of an increase in the local concentration of DMS consecutive to the formation of a hydrophobic pocket at a close contact site between RNA and protein, as previously proposed for specific protein-DNA interactions (17).

It may be noteworthy that data about the accessibility to chemical probes of some nucleotides of yeast 18S rRNA in 40S subunit have been reported in two previous studies (18, 20). In the first work (18), the structural probe was also DMS but a very limited number of nucleotides were found accessible in the 5'domain as compared to the present study : since the mapping procedure in that work involved aniline-induced chain cleavages, only the most reactive N-7 methylations of G (which are only indicative for tertiary interactions and are not "seen" in our approach) and N-3 methylations of C (indicative for base-pairings and "seen" in this work) were identified. For region 1-320, only one C (position 70) was found readily reactive by these authors. Our present data agree well with the accessibility of this residue (located in the loop of helix 5), although a few additional Cs also appear plainly reactive in our conditions.

As for the second work (20) using kethoxal modifications of guanine, 5 accessible Gs were detected in the 5' domain (positions 6, 81, 83, 115 and 264), which are not seen as reactive in our study. These differences may simply reflect the fact that the two chemical probes recognize different parts of the purine molecule as discussed elsewhere (37).

Finally, DMS probing followed by reverse transcription mapping can represent an effective procedure for rapidly assessing the secondary structure status of any kind of bases along an RNA sequence thereby providing a useful means for better assessing the "nativeness" of isolated RNA, according to the isolation procedure, and evaluating the potential contribution of bound proteins. As mentioned above, it must be stressed that the interpretation of the DMS accessibility data by themselves may, in some cases, remain equivocal. However, a number of intrinsic ambiguities can be solved by associating this method with techniques such as psoralen cross-linking (36) or direct isolation of base-paired RNA strands (38) and by reference to the indications provided by the comparative analysis of sequence data.

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*To whom correspondence should be addressed

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