Probing the structure of RNAs in solution

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SUMMARY

During these last years, a powerful methodology has been developed to study the secondary and tertiary structure of RNA molecules either free or engaged in complex with proteins. This method allows to test the reactivity of every nucleotide towards chemical or enzymatic probes. The detection of the modified nucleotides and RNase cleavages can be conducted by two different paths which are orientated both by the length of the studied RNA and by the nature of the probes used. The first one uses end-labeled RNA molecule and allows to detect only scissions in the RNA chain. The second approach is based on primer extension by reverse transcriptase and detects stops of transcription at modified or cleaved nucleotides. The synthesized cDNA fragments are then sized by electrophoresis on polyacrylamide:urea gels. In this paper, the various structure probes used so far are described, and their utilization is discussed.

INTRODUCTION

A wide range of functions are devoted to RNA molecules in the cell. RNAs are involved in all steps of protein synthesis, by storing the genetic information (messenger RNA), by participating in the structure of the mRNA decoding machinery (the ribosome), by carrying the aminoacids onto the ribosome (tRNAs). This multimolecular mechanism requires specific and coordinated RNA-RNA and RNA-protein interactions. Besides these canonical roles, several unexpected new functions for RNAs have been recently reported, e.g. enzymatic activity in RNA splicing and maturation (1-7), priming of DNA synthesis in mitochondria (8), priming reverse transcription (9), biosynthesis of the heme (10). Obviously, the three-dimensional structure of RNAs determines many of their biological activities. Thus, the precise mapping of secondary and tertiary structure features are of prime importance for a detailed understanding of the RNA functions.

X-ray crystallography of RNAs or ribonucleoprotein complexes is required to solve RNA structures at high resolution. However, up to now only tRNAs have yielded crystals able to diffract at high resolution (11-18). Valuable information can also be brought on RNAs in solution by physical approaches such as X-ray and neutron scattering or NMR studies. However, all these techniques suffer essentially by the requirement of large amounts of highly purified material.

Elaboration of secondary structure models results from the combination of several approaches. Two of them are based: (i) on sequence comparison of homologous RNAs from different organisms covering a large range through the evolution; (ii) on the search of the most favorable structure by using thermodynamic parameters. For this purpose, computer programs have been elaborated (19, 20). A third general approach to probe the secondary structure of RNAs is experimental, including: (i) the isolation and analysis of base paired RNA fragments obtained by mild RNase digestion under native conditions; (ii) intra-RNA crosslinking techniques; (iii) binding of complementary oligodeoxyribonucleotides to single-stranded exposed RNA regions; (iv) determination of splitting positions produced by nucleases specific to single-stranded or double-stranded regions (for a review, see reference 21). However, the only possibility to get a detailed insight into the folding of the RNA is to test the reactivity of every nucleotide towards specific structure probes. The goal of this paper is to give an overview of the various experimental approaches available to map the structure of RNAs in solution at nucleotide resolution.

I. OVERALL STRATEGY
A. Principle

In the first step, the RNA or RNA-protein complex is subjected to limited RNase hydrolysis or chemical modification using specific structure probes. Enzymatic cleavages or chemical modifications are introduced at a statistical and low level (less than one cut or modification per molecule). Controls are incubated in parallel under the same conditions but in the absence of the probe. Two different methodological paths are used to identify RNase cleavages and modified nucleotides. Both methods derive from gel sequencing methodology of nucleic acids. The choice between the two paths is orientated both by the length of the studied RNA molecule and by the nature of nucleotide positions to be probed. The first path uses end-labeled RNA molecules but is limited to molecules containing less than 200 nucleotides or to the terminal 200 residues of larger molecules, in consequence of the resolution of sequencing gels. This approach allows to detect only scissions of the RNA chain (arising from RNase hydrolysis or chemical treatment). The second approach using primer extension detects stops of transcription at modified or cleaved nucleotides. This technique allows to probe internal regions of large RNA molecules and is recommended even for small molecules to map chemical modifications at Watson-Crick positions that cannot be revealed by chemical scission.

B. Detection of cleavages on end-labeled RNAs.

In this approach, the RNA molecule is labeled at its 5' or 3'-end prior to RNase hydrolysis or chemical modification. Labeling at the 5'-end is achieved by transferring the γ-phosphate from $[\gamma^{32}P]$-ATP to the terminal OH group of the RNA, using T4 polynucleotide kinase (22, 23). When a 5'-phosphate is present, the RNA has to be dephosphorylated using alkaline phosphatase prior labeling (24). Alternatively, labeling can be done by an exchange reaction

between the γ-phosphate of [γ^{32}P]-ATP and the 5'-phosphate of the RNA catalyzed by T4 polynucleotide kinase (25). Labeling at the 3'-end is achieved by adding [5'-^{32}P]-pCp at the 3'-OH of the RNA with T4 RNA ligase (26). In the particular case of tRNAs, labeling can be done by removing the 3'-terminal CCA$_{OH}$ by limited phosphodiesterase digestion and by restoration of the CCA-end using tRNA nucleotidyltransferase in the presence of CTP and [α^{32}P]-ATP (27, 28). This has the advantage to yield homogeneous populations of tRNA molecules keeping their aminoacylation capacity unaltered.

The labeled RNA is then subjected to the attack of the probe under statistical conditions, in the presence of carrier RNA (added in order to control the probe:RNA ratio). Reaction is stopped and the RNA is subjected to phenol extraction when necessary. Chemical modification must be followed by a further chemical treatment leading to strand scission at modified nucleotides. The generated RNA fragments are then sized by electrophoresis on polyacrylamide-urea gels followed by autoradiography. Determination of the size of the fragments is facilitated by running on the same gel an alkaline hydrolysis ladder and a sequencing reaction (e.g. a RNase T1 ladder giving the position of G residues).

C. Primer extension

This method was initially developed by HuQu et al. (29) for probing the structure of large RNA molecules. The unlabeled RNA is first subjected to statistical RNase digestion or chemical modification. After stopping the reaction, the RNA is phenol extracted when required and then hybridized with an oligodeoxyribonucleotide complementary to a chosen sequence in the RNA. This oligomer which can be synthesized or prepared by DNA restriction, is used as a primer for reverse transcriptase. Elongation proceeds from the 3'-end of the primer in the 5'-direction, in the presence of the four dNTPs. Prematurely terminated cDNAs are obtained instead of fully elongated chains which are normally synthesized on an unreacted RNA template. These chain terminations are the result of scissions of the template or of chemical modifications. Indeed, modification at one of the Watson-Crick positions blocks the progress of reverse transcription at the nucleotide preceding the modified residue. This is the case for the modification of N1-A and N3-C with dimethylsulfate (DMS), for N1-G and N3-U with 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluene sulfonate (CMCT) and for N1-G and N2-G with β-ethoxy-α-ketobutyraldehyde (kethoxal). As for non-Watson-Crick positions, the effect may depend upon the chemicals. Methylation of N7-G with DMS does not affect primer extention. In that case, aniline treatment is required to induce a cleavage at the corresponding phosphodiester bond. Carbethoxylation of N7-A with diethylpyrocarbonate (DEPC), which results in the opening of the imidazole ring, appears to be sufficient to stop reverse transcription.

Labeling of the synthesized cDNA chains can be achieved by two different procedures. In the first one, the primer is labeled at its 5'-end in the presence of [γ^{32}P]-ATP prior hybridization. This procedure is particularly suited when only small amounts of the RNA template are available, since very high specific activities can be obtained. In the second procedure, the primer

is unlabeled and elongation is achieved in the presence of $[\alpha^{32}P]$-dATP or $[\alpha^{35}S]$-dATPαS. This labeling procedure is particularly recommended when the yield of hybridization is low since excess of primer can be used without masking the first elongation products. In both cases, the resulting labeled cDNA chains are sized at sequence resolution by analyzing the sample in parallel to a set of the four dideoxyribonucleotides sequencing reactions (30) carried out on unmodified RNA using the same DNA primer.

One limitation of the primer extension technique is directly related to the property of the reverse transcriptase to be stopped by modifications at Watson-Crick positions. Thus, the presence of naturally modified nucleotides in RNA (such as m^2G, m^6_2A) blocks the elongation.

Pauses of reverse transcription are also observed, reflecting the difficulty of the transcriptase to melt particular features of the folding of the RNA template. Elongation controls on unreacted RNA have to be run in parallel in order to detect such natural pauses, and also to detect breaks in the template (e.g. in CpA or UpA sequences).

II. ENZYMATIC AND CHEMICAL PROBES

A. Enzymatic probes

The accessibility of RNA molecules can be probed by nucleases specific to single-stranded or structured regions. The various RNases described here are presented in Table I with their molecular weight and their specificity.

1. RNase T1

RNase T1, from *Aspergillus orizae,* specifically splits internucleotide bonds adjacent to the 3'-phosphate of unpaired guanosine residues in RNA, with the intermediary formation of guanosine 2'-3'-cyclic phosphate. The hydrolysis generates fragments with a 3'-phosphate (31, 32). Two pH optima, one at 7.5 and a second one at 4.5, have been determined (33). At pH 4.5, the presence of 7M urea increases the enzyme activity (34). It was shown that positions N1 and O6 of the guanine interact through hydrogen bonds with the nuclease (35, 36). The naturally modified m^1G and m^7G in RNAs are not recognized by the enzyme. Utilization of RNase T1 for structure probing is described in references 37-44.

2. RNase U2

RNase U2, from *Ustilago sphaerogena,* preferentially cleaves 3'-5' phosphodiester bonds of unpaired adenines in RNA. However, its specificity is not absolute (A>G>>C>U) (45). The optimum pH is 4.5 and the enzyme is still active in the presence of 7M urea (34). The mechanism of splitting is similar to that proposed for RNase T1. The hydrolysis conditions have been adapted at neutral pH for probing single-stranded adenines (43, 44).

3. RNase Cl3

RNase Cl3, from chicken liver cleaves almost exclusively unpaired cytidines and generates fragments with a 3'-terminal phosphate (46, 47). The enzyme also cleaves the RNA chains at adenosines and uridines but long incubation times and high enzyme concentrations are required.

The optimum pH is dependent on the type of buffer used in the reaction (in Tris-HCl buffer, the optimum pH is 7.5). The activity of the RNase is enhanced in the presence of spermine and of magnesium ions (46). For applications, see reference 40.

4. RNase T2

RNase T2, from *Aspergillus orizae*, is a single strand-specific endonuclease with a preference for adenosine residues (31). The mechanism of splitting is similar to that proposed for RNase T1. The optimum pH is 4.5 but the enzyme is still stable around neutral pH (45). RNase T2 is inhibited by heavy metal ions, especially Cu^{++}. For applications, see references 42 and 48-50.

5. Nuclease S1

Nuclease S1, from *Aspergillus orizae* , is a single strand-specific endonuclease stimulated by Zn^{++} (51). It degrades RNA as well as DNA and yields fragments with a 5'-phosphate. Nuclease S1 has been extensively used at its optimal pH (4.5) for probing unpaired RNA regions (e.g. references 37, 38, 40, 41 and 52). Hydrolysis can also be conducted at neutral pH, but high concentrations are required (43, 44).

6. Neurospora crassa nuclease

This single-strand specific nuclease contains Co^{++} as a prosthetic group. It cleaves RNA or DNA and generates fragments terminated by a 5'-phosphate (53). At low ionic strength and at pH 8.0, the nuclease is not sequence specific (54, 55). The optimum pH is 7.5-8.0 and the enzyme is still active at 65°C. The presence of EDTA inhibits its activity. For application, see reference (56).

7. RNase V1

RNase V1, an endonuclease from cobra *Naja naja oxiana* venom, preferentially cuts double-stranded or structured regions without base specificity and generates fragments with a 5'-phosphate (57). It has been shown that the minimum size of the RNA substrate is 4 to 6 nucleotides and that 3 to 4 ionic interactions are formed between positive groups of the protein and phosphates of the RNA (58). RNase V1 also cleaves single-stranded regions but in a stacked conformation (58, 59). The enzyme is active in the pH range of 4-9. It requires magnesium (at least 1mM) and the addition of EDTA results in more than 90% of inhibition (60). For applications, see e.g. references 42-44 and 61-65.
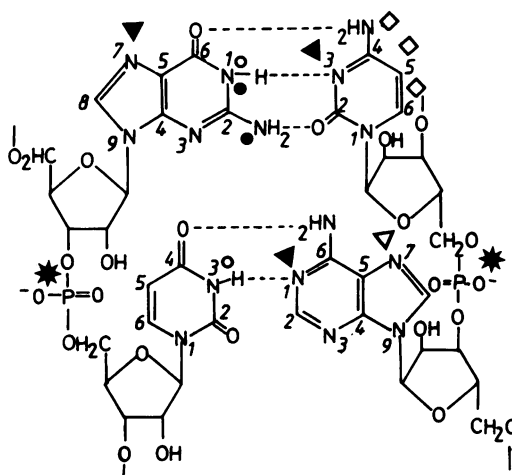
B. Chemical probes

The different chemical probes and their respective targets are presented in Table I with the mode of detection of the resulting modifications indicated. The target positions are shown on a Watson-Crick base-pairing scheme in Fig.1. In addition to these classical interactions, other non-canonical interactions may occur, essentially participating in the tertiary folding of the RNA molecule. Some of these interactions, already evidenced in nucleic acids (18, 11, 12, 66-68) are listed in Table II. This list is not restrictive and other hydrogen bonding possibilities probably

probes	molecular weight	specificity	detection A	B
RNase T1	11,000	unpaired G	+	+
RNase U2	12,490	unpaired A>G	+	+
RNase CL3	16,800	unpaired C>>A>U	+	+
RNase T2	36,000	unpaired N	+	+
nuclease S1	32,000	unpaired N	+	+
N.c. nuclease*	55,000	unpaired N	+	+
RNase V1	15,900	paired or stacked N	+	+
DMS	126	N3-C	s	+
		N1-A	-	+
		N7-G	s	s
DEPC	174	N7-A	s	+
CMCT	424	N3-U	-	+
		N1-G	-	+
kethoxal	148	N1-G, N2-G	-	+
bisulfite	104	unpaired C-->U	s	s
ENU	117	Phosphates	s	s
MPE-Fe(II)	780	paired N	s	s

Table 1 : *Chemical and enzymatic probes.* The molecular weight and the specificity of the various probes are given and the possible way to detect the cleavages and modifications is indicated : (A) detection of cleavages on end-labeled RNA molecule; (B) primer extension method. (+) means that the concerned detection method can be used, (-) means that the concerned detection method cannot be use, (s) means that a chemical treatment is necessary to split the ribose-phosphate chain ; (*) *Neurospora crassa* nuclease.

Figure 1 : *Canonical Watson-Crick interactions and target positions of the chemical probes* (▼) DMS; (▽) DEPC; (o) CMCT; (◇) bisulfite ; (●) kethoxal; (✳) ENU.

Table II : Non-canonical interactions in nucleic acids

| interaction	Hydrogen bond	observed in
A-U	(N7, N6)-(N3, O2)	tRNA^{Phe}, tRNA^{Asp} : A14-U8, A58-T54 (11, 12, 18)
A-U	(N1, N6)-(N3, O2)	tRNA^{Asp} : A15-U48 (18)
A-G	(N1, N6)-(N1, O6)	tRNA^{Phe} , tRNA ^{Asp} : A44-G26 (11, 12, 18)
A-G	(N 6)-(N 7)	tRNA^{Asp} : A46-G22-U13 (18)
A-A	(N6, N7)-(N7, N6)	tRNA^{Phe}, tRNA^{Asp} : A9-A23-U12 (11, 12, 18)
G-G	(N3, N2)-(N2, N3)	ß-dodecamer (66)
G-G	(N6)-(N7, O6)	tRNA^{Asp} : G45-G10-U25 (18)
G-G	(N6)-(O6)	tRNA^{Phe} : G45-G10-C25 (11, 12)
G-G	(N1, N2)-(N7, O6)	tRNA ^{Phe} : m^{7}G46-G22-C13 (11, 12)
U-U	(N3, O4)-(O2, N3)	tRNA^{Asp} : U35-U35, anticodon-anticodon interaction (18)
C-C	(N4, N3)-(N3, O2)	tRNA^{Gly} : C35-C35, anticodon-anticodon interaction (67)
C-C	(N4, N3, O2)-(O3, N3, N4)	poly C (68)

exist. The mechanism of reaction of the various probes is illustrated in Fig. 2a-f.

1. Dimethylsulfate (DMS)

DMS reacts primarily with N7-G, N1-A and N3-C at neutral pH (69, 70) (Fig. 2 a). This reagent was first used for DNA and RNA sequencing (71, 72). Its use for probing the conformation of RNAs was first described by Peattie and Gilbert (73). Since that time, it has been largely used (e.g. references 41, 43, 44, 50, 65 and 74-77).

i) Guanine reaction . The addition of a methyl group on the N7 position leads to the presence of a positive charge which perturbs the electron resonance of the purine ring (69, 70, 78). The perturbed 7,8-double bond of the alkylated ring can easily be reduced in a diluted sodium borohydride solution at pH 8.3 (79). The resulting m^{7}dihydroguanosine, which hydrolyzes partially or completely to unknown products, provides a site for aniline induced strand scission (shown in Fig. 2c) (79). This reaction maps free N7-G. The absence of reactivity provides an indication for the involvement of this position in non-canonical base pairs or in coordination with ions (such as magnesium). The modification is also sensitive, to a certain extent, to base stacking. It was proposed by Romby et al. (77), from comparative analysis of various tRNAs in solution, that guanines in a regular A-helix are reactive when flanked by pyrimidines. They also show that slight distorsion of the helix may alter this rule. However, in the case of 16S rRNA, no apparent rule could easily be deduced (44).

ii) Cytosine reaction. Unpaired cytosines are methylated at their position N3. The resulting methyl-3 cytosine can be detected directly by primer extension but a further chemical treatment is required when end-labeled RNAs are studied. The sensitivity of methyl-3 cytosine to mild hydrazinolysis is then used to provide a site for aniline scission. Some uridine cleavage may also appear, as a result of the hydrazine step. This latter reaction does not reflect RNA conformation, since the RNA is denatured at the pH of the hydrazine treatment. Note that gel electrophoresis patterns are less clean with 5'-end labeled than with 3'-end labeled RNA. This

DMS

A

DEPC

B

aniline scission

C

CMCT

Kethoxal

R = CH₃CH(OC₂H₅)

D

Bisulfite

E
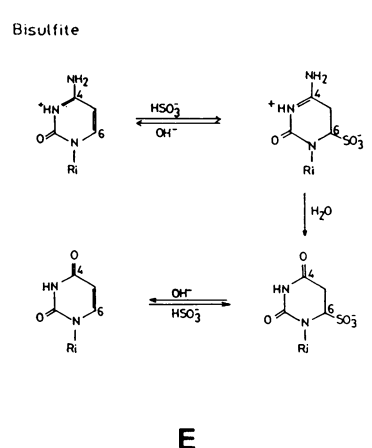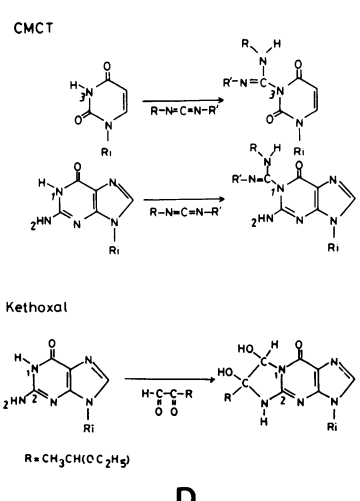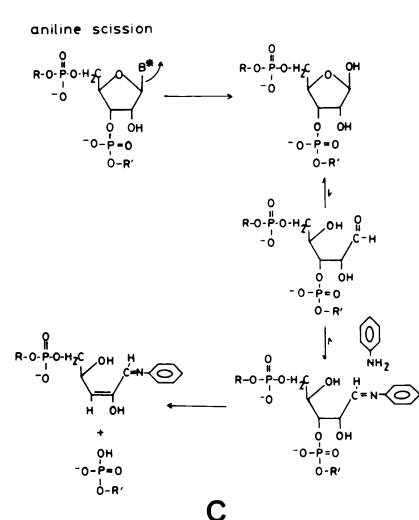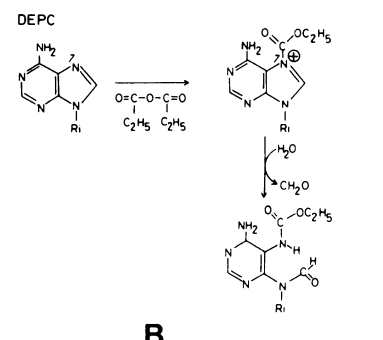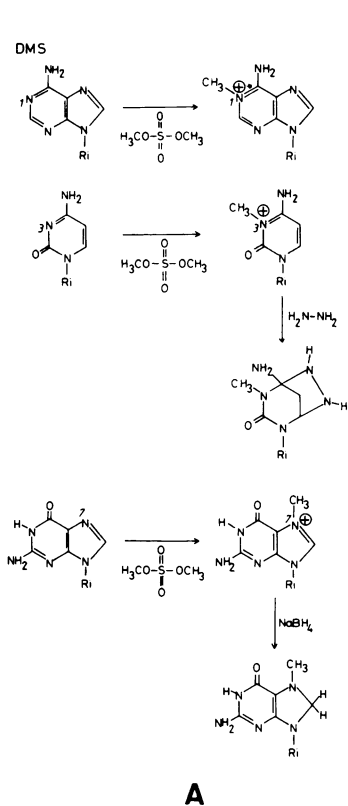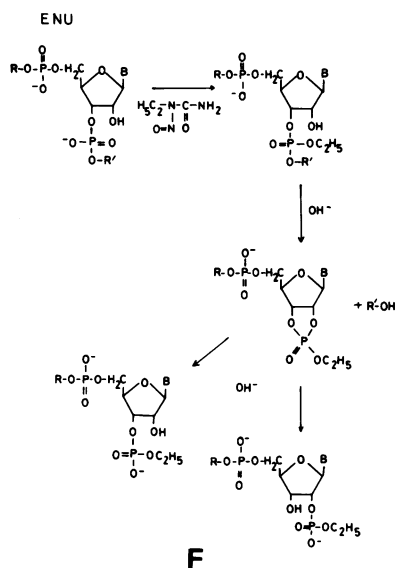
Figure 2 : _Mechanism of the chemical reactions._ (A) DMS alkylation of N1-A, DMS alkylation of N3-C followed by hydrazine treatment and alkylation of N7-G followed by sodium borohydride reduction; (B) Carbethoxylation of N7-A with DEPC; (C) aniline strand scission : after the hydrolysis of the glycosidic bond between the modified base and the ribose, the exposed ribose forms an open-ring tautomer. The free aldehyde group of this tautomer reacts with aniline to form an aldimide, and the 3'-phosphoester bond is cleaved via a ß-elimination mechanism; (D) CMCT modification at N3-U and N1-G, and kethoxal modification at N1-G and N2-G; (E) Bisulfite conversion of unpaired cytosine to uridine; (F) Alkylation of phosphate by ENU followed by alkaline treatment.

most likely results from a side-reaction of the hydrazine with the exposed ribose moiety at the 3'-end of the 5'-end labeled fragment.

iii) Adenine reaction . Since no chemical splitting of the RNA chain at methyl-1 adenine exists, this modification can only be detected by the use of the primer extension method. This reaction allows to map single-stranded adenines.

2. _Diethylpyrocarbonate (DEPC)_

The N7 atom of adenosine is particularly susceptible to carbethoxylation (80-82) at neutral pH. The modification destroys the resonance of the heterocyclic ring and the imidazole ring opens between atoms N7 and C8 (Fig. 2b) (80), thereby creating a site for aniline strand scission (72). This probing reaction monitors the involvement of N7-A in tertiary interactions (as described for N7-G alkylation to DMS). Due to the higher molecular weight of the adduct, DEPC is more sensitive to stacking than DMS and all adenines in helices are unreactive. For applications, see e.g. references 43, 44, 73, 74, 77, 83 and 84.

Carbethoxylation at N7-G at acidic pH and at N3-U in slightly basic solution have also been

described to occur (81). Carbethoxylation of N3-U has been observed at neutral pH independently of salt concentration (43), and cytidines have also been found to be reactive at high salt concentration (84). However, it is not yet clear which structural parameters lead to the reactivity of pyrimidines.

3. 1-cyclohexyl-3- (2-morpholinoethyl) carbodiimide metho-p-toluene sulfonate (CMCT)

CMCT primarily reacts with N3-U and N1-G at pH 8.0 in the order N3-U>N1-G (Fig. 2d) (84-86). The reaction does not work at neutral and acidic pH. CMCT is used to map unpaired uridines and guanosines. For applications, see references 43, 44, 50, 65 and 76. Thymidine also reacts with CMCT, but to a lower extent. Pseudouridine forms with the reagent either a mono or disubstituted derivative depending on experimental conditions (86).

4. β-etoxy-α-ketobutyraldehyde (kethoxal)

Kethoxal, a glyoxal derivative, reacts specifically with unpaired guanosines to form a new ring involving the N1 and N2 positions of the guanine ring and both carbonyl groups of kethoxal (Fig. 2d) (87-88). The kethoxal-guanine adduct is stable under slightly acidic solution but is readily decomposed into its components if kept at basic pH (87). The presence of borate ions is found to stabilize the adduct. For applications, see references 50, 65 and 76.

5. Bisulfite

Bisulfite modification converts unpaired cytosine to uridine (for a review, see reference 89). Bisulfite reacts with cytosine forming 5,6-dihydrocytosine-6-sulfonate (Fig. 2e). At high concentration of bisulfite (>1M) and acidic pH (pH 5-6), the formation of the cytosine derivative facilitates nucleophilic substitution at the exocyclic amino group, allowing the conversion of 5,6-dihydrocytosine- 6-sulfonate to 5,6-dihydrouracyl-6-sulfonate. The bisulfite moiety can then be removed by treatment with mild alkali. Cytidine to uridine conversion can be monitored by the uridine specific sequencing reaction using hydrazine (72). For applications, see references 90-91.

6. Ethylnitrosourea (ENU)

ENU is a N-nitroso alkylating reagent which has an affinity for the phosphate group oxygens of nucleic acids, in contrast to other alkylating reagents (such as DMS) which primarily alkylates ring nitrogens (Fig. 2f) (92-94). In the case of RNA, the resulting phosphotriesters are unstable and are easily split by mild alkaline treatment (95). Alkylation specifically maps phosphates non engaged through hydrogen bonds in tertiary interactions or non involved in cation coordination (28, 96). Note that phosphates in helices are fully reactive to ENU. For applications, see references 43 and 96-100.

7. Methidiumpropyl-EDTA. Fe(II) [MPE-Fe(II)]

MPE is a methidium intercalator moiety tethered to EDTA. Upon addition of Fe(II) and a reducing agent such as DTT, the ferrous ion binds EDTA and generates short-lived radicals that cleave nearby phosphodiester bonds (101-102). The reagent binds to poly(A)-poly(U) with about the same affinity as the related ethidium intercalator, it selectively binds double helical in

preference to single-stranded RNA, and when complexed with Fe(II) it readily cleaves the RNA backbone (103). The reaction works at pH 7.5 in the presence of 0.1 M NaCl (1034. MPE-Fe(II) is a small molecular weight probe, having a great reactivity towards base-paired regions. For applications, see references 103 and 104.

III. EXPERIMENTAL CONDITIONS

Since the optimal conditions vary with the different probes, the possibility exists that subtle conformational changes may occur under different incubation conditions. Therefore, probing the conformation of RNAs requires strictly defined buffer conditions (pH, ionic strength, magnesium concentration and temperature). For this purpose, the probe:RNA ratio must be adapted. This is the case for nuclease S1 and RNase U2 which have their pH optimum at 4.5 and thus were adapted at pH 7.5. Indeed, a pH effect has been observed on the tRNA-like structure from turnip yellow mosaic virus (40) when using nuclease S1. Regarding the chemical probes, note that CMCT requires to be used at pH 8.0 and bisulfite at pH 5.0.

The chemical reactions can be conducted under different conditions : under native conditions (at low temperature, in the presence of magnesium and monovalent salt), under semi-denaturing conditions (at low temperature, in the presence of EDTA) and under denaturing conditions (at high temperature, in the presence of EDTA). This allows to estimate the degree of stability of the different helical domains of RNAs (e.g. 43, 44, 73 and 77). Tertiary interactions which are less stable than canonical Watson-Crick interactions are expected to melt under semi-denaturing conditions. Since chemical probes can react at different temperatures, it is possible to follow the thermal transitions of the RNA molecules (74). As a general rule, buffers containing amino groups must be avoided when using chemical probes.

It might be noticed that the RNA is quite stable after the initial modification by DMS, DEPC and ENU under the conditions used for structure mapping. This means that the RNA molecule can be modified and then tested for biological activity. The various probes do not only allow to investigate the higher-order structure of RNAs but can also be used to map protein binding sites. In the later case, control of the stability of the complex in the presence of the probe has to be done, because chemical reagents may also react with proteins (see § IV).

IV. ADVANTAGES AND LIMITATIONS OF THE PROBES
A. Enzymes

Due to their bulky size, nucleases are sensitive to steric hindrance, especially in the presence of bound proteins. They can also be sterically blocked by particular folding of the RNA.

Nucleases have optimum hydrolysis conditions which may differ from one another. As it is important to work under strictly defined conditions, nucleases are not necessarily used under their optimum conditions.

Enzymatic cleavage at a particular position may alter the structure of the RNA, thereby

inducing artefact cuts on the same RNA chain. Such cleavages, designated as "secondary cuts" can be easily discriminated from primary cuts on end-labeled RNA by comparing hydrolysis patterns of 5' and 3'-end labeled molecules. Secondary cuts which are weaker than primary ones, are only observed in one of the two end-labeled species. When primer extension is used, this control is not possible since elongation only proceeds in one direction.

B. Chemicals

Due to their small size, chemical probes are weakly sensitive to steric hindrance and allow to get a detailed insight at atomic level. The various nucleotide positions do not react evenly with the probes, thus N3-C reacts slower than N1-A with DMS, and N1-G slower than N3-U with CMCT. It must also be reminded that the reactivity of N7-A towards DEPC is more sensitive to stacking than N7-G towards DMS.

When nucleic acids are probed with chemicals, it is often implicitly assumed that the probes map stereochemical accessibilities. Strictly speaking, that is an incorrect assumption since the probes map chemical reactivity of groups which cannot necessarily be correlated directly with geometrical accessibility. Indeed, the reactivity is largely influenced by the electrostatic environment of the molecule. This problem, which may represent a limitation of the chemical mapping approach, has been discussed by Lavery and Pullman (105). These authors introduced a new theoretical index, combining steric and electrostatic factors (computation of ASIF "Accessible Surface Integrated Field" indexes) (105-106). In general, the more negative the ASIF's are, the more likely are the chemical modifications to occur. It is interesting to note that a good agreement has been found between theoretical calculation of ASIF indexes (based on X-ray structure) of tRNA^{Asp} and tRNA^{Phe} and the observed reactivity towards ENU (96).

Chemical reagents often react not only with nucleic acids but also with proteins. Thus, DEPC strongly reacts with histidine and cysteine residues (82), and ENU with cysteines (107). This may limit the use of these reagents when mapping RNA-protein complexes. Therefore, controls must be made to test the stability of complexes in the presence of the chemical reagent. Large excess of protein is sometimes required to protect the complex against destabilization.

V. APPLICATIONS

In order to illustrate the use of the techniques described in this paper, two distinct examples will be given. The first one shows the detection of ENU alkylated phosphates on a end-labeled molecule. The second example illustrates the detection of chemical modifications by the primer extension method.

A. Mapping of the phosphates of yeast tRNA^{Asp} and correlation with the crystal structure.

The reactivity of phosphates towards ENU alkylation was used to probe the tertiary structure of yeast tRNA^{Asp} in solution and the results were correlated with the crystal structure of the tRNA (96). A representative example of a gel fractionation pattern of alkylated 5'-end labeled tRNA^{Asp} is shown in Fig. 3A. The position of phosphates showing a decreased reactivity
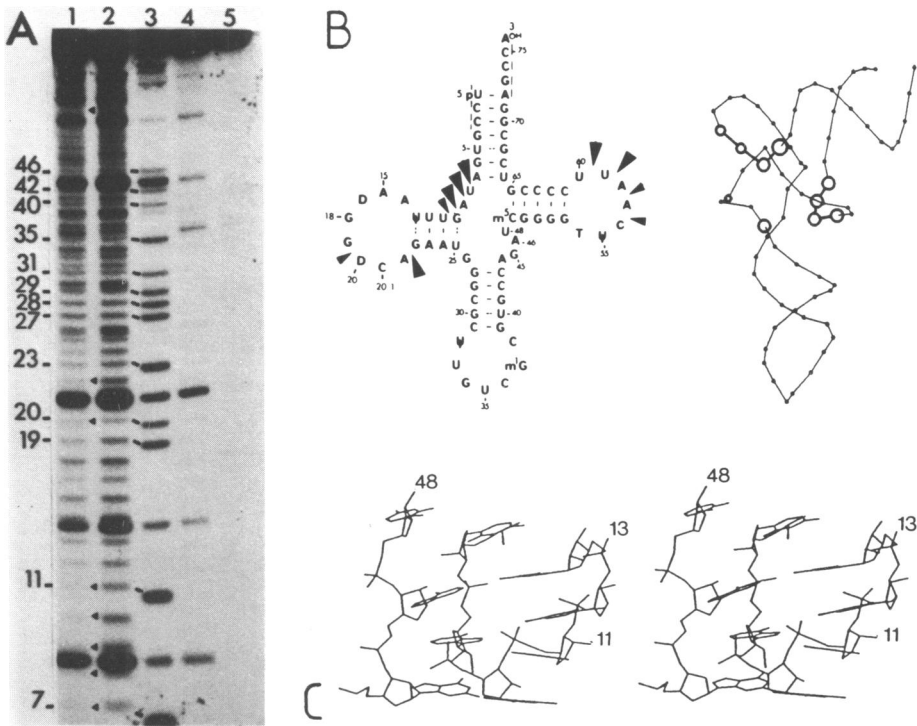
Figure 3 : Modification of phosphates of tRNA^Asp with ENU. (A) Gel electrophoresis fractionation of products resulting from the alkylation of 5'-end labeled tRNA. (1, 2) : alkylation under native and denaturing conditions, respectively; (3) : RNase T1 ladder; (4) : incubation control of tRNA under native conditions in the absence of ENU; (5) : tRNA control without any incubation. (B) Cloverleaf (left) and 3-dimensional (right) structures of tRNA^Asp with the position of phosphates protected from ENU. (C) Stereoscopic views of the structural environment of phosphates 8-11 showing a magnesium site in the crystallographic structure of tRNA^Asp (18). Coordinates of yeast tRNA^Asp are those from Dumas et al. (116). The stereoscopic views were taken on the PS 300 of Evans and Sutherland using the program FRODO (117, 118).

towards ENU in native tRNA^Asp vs denatured tRNA, is represented in Fig. 3B. They are all located in the hinge region where the two helical domains of tRNA join to form the characteristic L-shaped structure. Phosphates 8-11 in the hinge region between the aminoacid and D-stems and phosphates 58-60 in the T-loop display a similar non-reactivity in different tRNAs (e.g. 96, 97 and 99). All these phosphates are involved in tertiary interactions with other nucleotides of the tRNA or are coordinated to magnesium ions. For instance, in the crystal structure of yeast tRNA^Phe it has been shown that phosphates 8, 9 and 11 interact through hydrogen bonds with one magnesium ion (108-110). A similar magnesium site has been suggested in yeast tRNA^Asp (18) explaining the observed protection from ENU. The conformation of phosphates 8-11 is
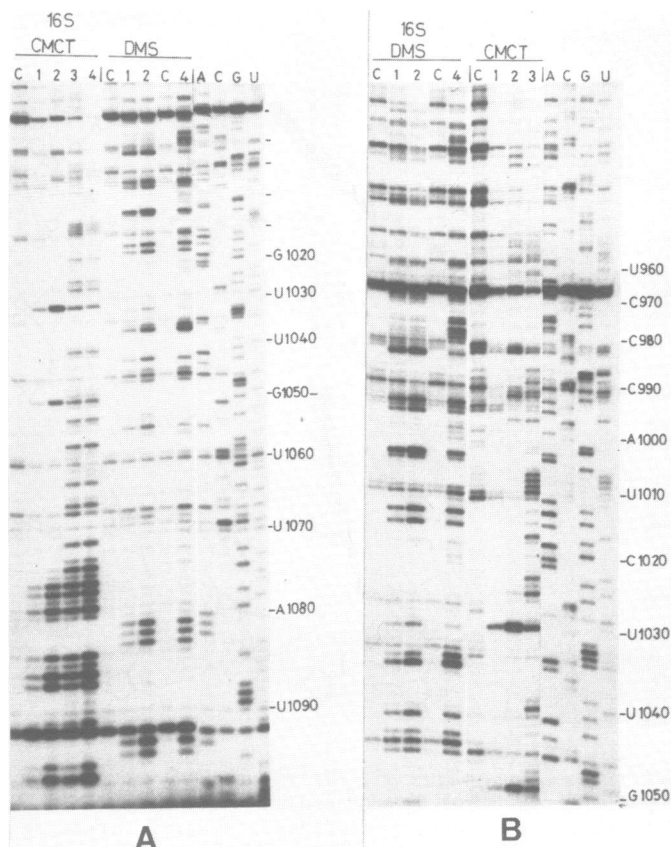
Figure 4 : *Gel electrophoresis fractionation of products resulting from CMCT and DMS modification of naked 16S rRNA*. Incubation was at 37°C in the appropriate buffer. Native conditions (in the presence of 20 mM MgCl$_2$ and 300mM KCl) : (C) incubation control; (1) CMCT : 15 min, DMS : 2 min; (2) CMCT : 30 min, DMS : 5 min. Semi-denaturing conditions (in the presence of EDTA 1 mM) : (3) CMCT : 5 min ; (4) CMCT : 10 min, DMS : 5 min. Lanes A, C, G and U are sequencing products generated in the presence of ddTTP, ddGTP, ddCTP and ddATP, respectively. Left : short migration; right : long migation. For more experimental details, see (44).

represented in Fig. 3C. This methodology was also successfully used to map the phosphates in contact with the aspartyl-tRNA^{Asp} synthetase. The example discussed here demonstrates that ENU is a convenient and efficient tool to probe phosphates involved in tertiary interactions or ion coordination.

B. <u>Higher-order structure of domain III in E. coli ribosomal 16S RNA</u>

The conformation of domain III in 16S rRNA has been extensively studied by using a variety of structure-specific probes (44). For most part, it has been shown that the results are consistent
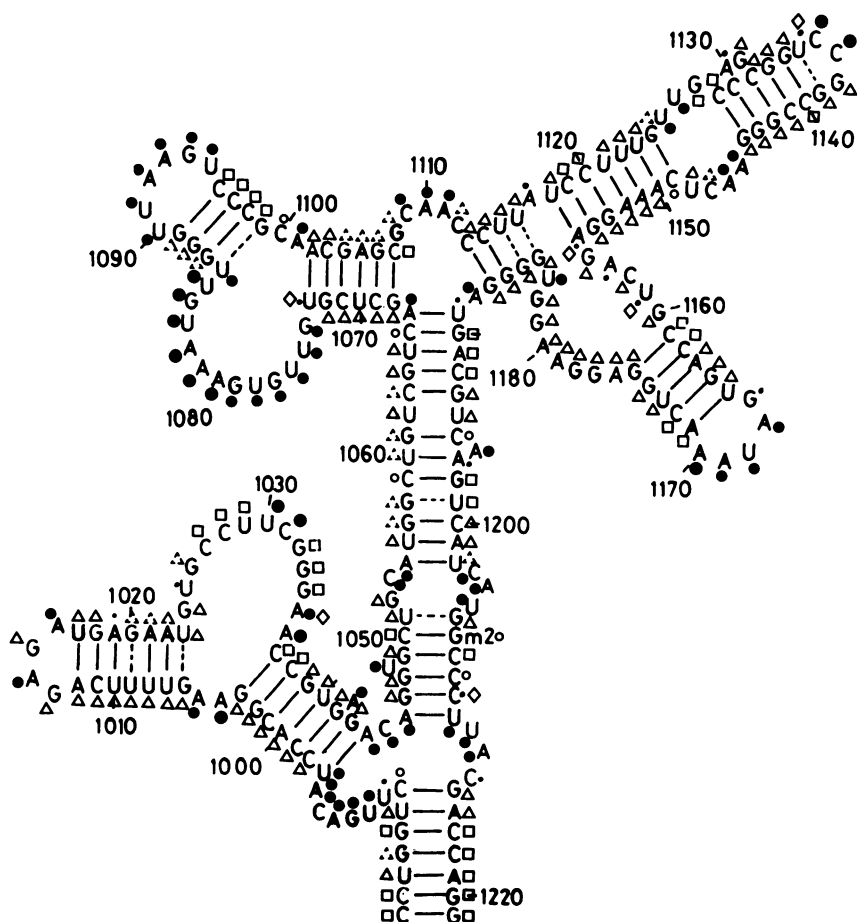
Figure 5 : Reactivity of Watson-Crick positions in naked RNA (nucleotides 984-1221) towards DMS (N1-A and N3-C) and CMCT (N1-G and N3-U). Reactive under native conditions : (●) strong hit, (●) moderate hit, (•) marginal hit; unreactive under native conditions but reactive under semi-denaturing conditions : (△) moderate hit, (⚠) marginal hit ; unreactive under both native and semi-denaturing conditions : (□); (◇) denotes increased reactivity under semi-denaturing conditions, as compared to native conditions. (○) denotes not determined positions.

with the most recently refined secondary structure model of 16S rRNA (111-112). Representative examples of gel electrophoresis fractionation of cDNA fragments resulting from primer extension on 16S rRNA modified with DMS and CMCT are shown in Fig. 4. The reactivity of nucleotides 984-1221 at Watson-Crick positions is summarized in Fig. 5. Nucleotides in exterior loops are reactive with a few notable exceptions. The stability of helices can be estimated from their observed reactivity under semi-denaturing conditions. Thus, helix

[1006-1012] / [1017-1023] appears totally unwound in the absence of magnesium. Several modifications of the pairing scheme have been brought by our study. The strong reactivity of GUU [1074-1076] and AUU [1081-1083] indicates that this region is more likely single-stranded than base-paired, as proposed elsewhere (112). Also we proposed a base pairing between AUG [1055-1057] with CAU [1200-1202] rather than with CAU [1203-1205]. Several nucleotides located in interhelical regions present an unexpected non-reactivity, suggesting that they can be involved in tertiary interaction. The methodology was also suitable for the study of 30S subunits, where extensive protection, due to the presence of ribosomal proteins, was observed. Strikingly, enhancement or new reactivity was also noted at numerous nucleotides, suggesting a local rearrangement of RNA induced by protein binding. This is fully discussed in reference 44. This particular example shows that the combined use of chemical probes and primer extension is a powerful approach allowing to probe any part of large RNA molecules.

VI. CONCLUDING REMARKS.

Probing the structure of RNA with a variety of structure-specific probes provides detailed data at nucleotide level. This approach is the most rigorous test available for probing secondary and tertiary RNA structure. When crystallographic data are available, it is possible to define a correlation between the crystal and solution structures. Such a study allows the prediction of structural rules that can help in the analysis of RNA for which no X-ray data are available. For these RNA molecules, structure probing permits to identify nucleotides involved in tertiary interactions. However, it is not possible to determine without ambiguity which nucleotides are interacting together in tertiary long-range interactions. Other experimental approaches, such as RNA-intramolecular crosslinking (see e.g. reference 113) are undoubtly required to provide complementary information. In fact, the goal of probing experiments is to constitute a data bank which may help when further information is available. The method also allows to study RNA-protein binding sites and to follow the conformation of RNA molecules under different functional states.

By combining experimental data resulting from structure probing of RNA in solution and specific information collected from the known crystallographic structures of tRNAs, it is possible to construct three-dimensional models of RNA. This has been done for the mammalian mitochondrial tRNA[Ser] (74), for the tRNA-like structure of turnip yellow mosaic virus (114) and for the 16S rRNA binding site of ribosomal protein S8 (43). Furthermore, it is now possible to produce large amounts of any RNA molecules by *in vitro* transcription (115) and to introduce mutation at selected positions. The effect of such mutations on the RNA conformation can be tested by chemical and enzymatic mapping. Thus, by combining mapping experiments with directed mutagenesis, genetic studies, crystallography and graphic modeling, it will be possible to define the precise relation between the structure and the function of RNA molecules.

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