

3. Anneal at 60° for 30 min.
4. Adjust the salt and buffer solution for the restriction enzyme to be used. Add the restriction enzyme and incubate under the recommended conditions.
5. Ethanol precipitate and analyze as described in steps 7 and 8 of the extension protocol (see Extension). Load the extension products, which have been treated with restriction enzyme, and the untreated extension products in adjacent PAGE lanes.

[26] Modification Interference Analysis of Reactions Using RNA Substrates

By LAURA CONWAY and MARVIN WICKENS

The modification interference method described in this chapter yields, in a single experiment, information equivalent to that obtained from the analysis of a large collection of point mutations.^{1,2} The method is analogous to that developed to analyze protein–DNA interactions by Siebenlist and Gilbert.³ Modification interference can identify those nucleotides in a RNA that are essential for any reaction of interest, provided that synthetic RNA can serve as a substrate in the reaction and that reaction substrates and products are separable.

General Method

We will describe in detail the use of two chemicals as modifying reagents: diethyl pyrocarbonate (DEPC), which carboxyethylates purine bases, and hydrazine, which removes pyrimidine bases (Fig. 1). Modification of RNA with either reagent renders the phosphodiester backbone susceptible to cleavage with aniline at the site of modification.⁴ We describe only DEPC and hydrazine modifications, since these modifications enable one to examine every nucleotide in a RNA; however, other modifications are also useful (see Other Modifications).

For modification interference (Fig. 2), an end-labeled RNA substrate is prepared *in vitro*. The end-labeled RNA substrate is modified using

¹ L. Conway and M. Wickens, *EMBO J.* **6**, 4177 (1987).

² B. Rymond and M. Rosbash, *Genes Dev.* **2**, 428 (1988).

³ U. Siebenlist and W. Gilbert, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 122 (1980).

⁴ D. A. Peattie, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1760 (1979).

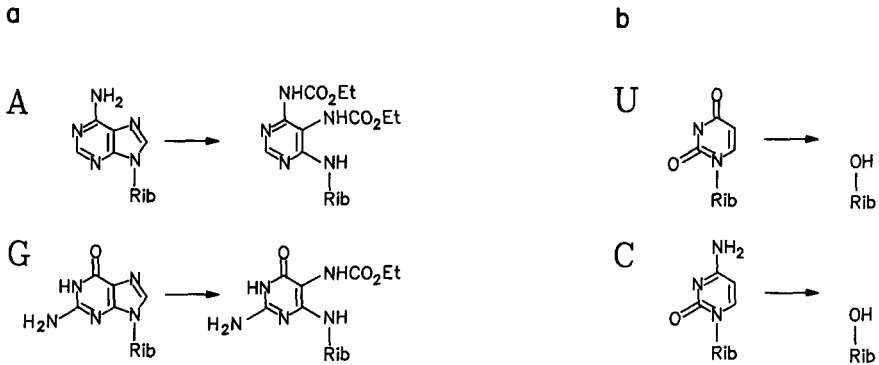


FIG. 1. Structure of bases following treatment with either (a) DEPC (purine modifications) or (b) hydrazine (pyrimidine modifications).

either DEPC or hydrazine, such that, on average, each molecule is modified only once. The modified transcripts are then used as substrate in the reaction of interest. RNAs that have successfully undergone the reaction are purified. The substrate and product RNAs are then cleaved with aniline and are analyzed by gel electrophoresis. Gaps in the sequencing "ladder" of the products correspond to sites which, when modified, prevent the reaction of interest.

Like point mutagenesis, the modification interference approach may not detect every nucleotide that is essential. Neither method will detect critical bases if more than one base must be changed to prevent the reaction; similarly, neither method will identify nucleotides that participate through their ribose moieties.

Materials

Enzymes

T4 RNA ligase was purchased from U.S. Biochemical (Cleveland, OH)

T4 polynucleotide kinase and SP6 polymerase were from New England Biolabs (Beverly, MA)

Calf intestinal alkaline phosphatase (molecular biology grade) was from Boehringer Mannheim (Indianapolis, IN).

Buffers

Buffer A: 50 mM Sodium acetate, pH 4.5, and 1 mM ethylenediaminetetraacetic acid (EDTA), pH adjusted with acetic acid

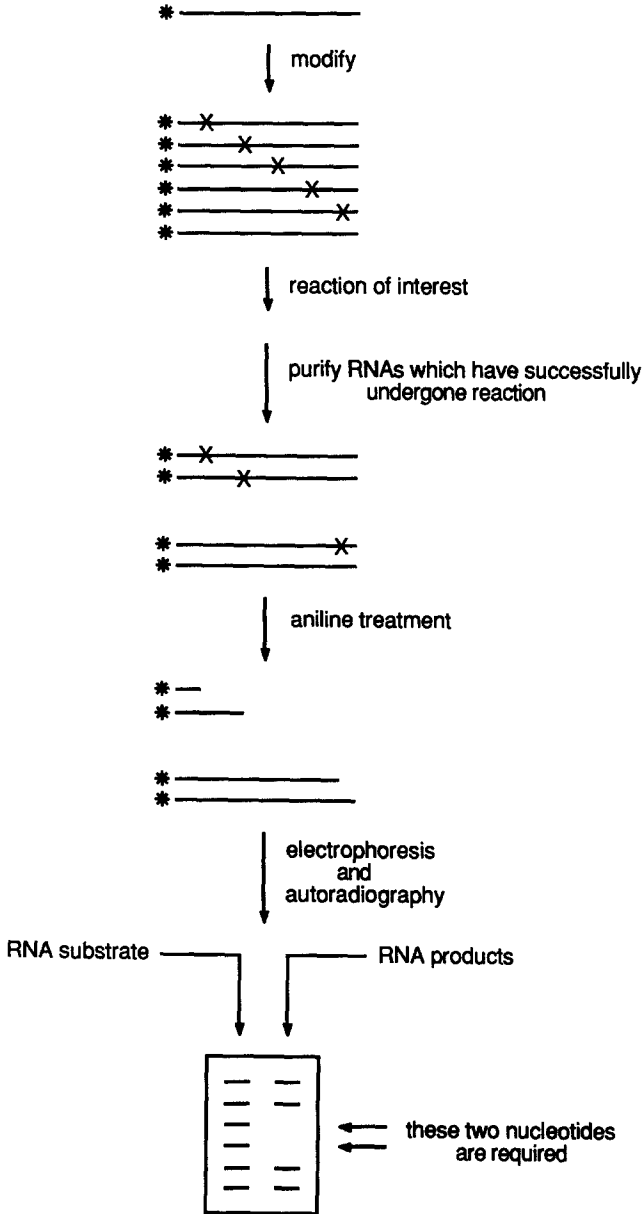


FIG. 2. Modification interference strategy. Modified nucleotides are indicated with X's. The asterisk (*) at the end of the RNA indicates that the RNA is end labeled. See the text for details.

Buffer B: 8 M Urea, 20 mM Tris-HCl, pH 7.9, 1 mM EDTA, 0.05% (w/v) xylene cyanol (XC), and 0.05% (w/v) bromphenol blue (BPB)

Buffer C: 20 mM Tris-HCl, pH 7.6, 1 mM EDTA, and 20 mM NaCl

Buffer D: 0.5 M Ammonium acetate, pH 6.5, 1 mM EDTA, and 0.1% (w/v) sodium dodecyl sulfate (SDS)

10× T4 polynucleotide kinase buffer: 0.5 M Tris-HCl, pH 7.6, 0.1 M MgCl₂, 50 mM dithiothreitol (DTT), 1 mM spermidine, and 1 mM EDTA

10× T4 RNA ligase buffer: 0.5 M Tris-HCl, pH 7.9, 0.15 M MgCl₂, and 33 mM DTT

Reagents

1 M Aniline, pH 3.8: Dilute pure aniline (11 M) with 0.3 M sodium acetate, pH 3.8. The aniline should be distilled once under nitrogen before use and stored at -20° in the dark

Phenol-chloroform: An equal volume of each, equilibrated with 0.1 M Tris-HCl, pH 7.9

Yeast RNA, DEPC, and dimethyl sulfoxide (DMSO) were from Sigma (St. Louis, MO)

Bovine serum albumin (BSA) and glycogen (both molecular biology grade) were from Boehringer Mannheim

Radionucleotides were from Amersham (Arlington Heights, IL)

Aniline and hydrazine were both Baker brands from VWR (Chicago, IL)

Sephadex G-25 (fine) beads were from Pharmacia (Piscataway, NJ)

All solutions were made with water that had been treated with DEPC.⁵ DEPC should be used within 1 week after DEPC is opened.

End-Labeling Methods

RNA is synthesized *in vitro* using an appropriate polymerase (see this volume, chapters [4] and [5]). The desired RNA species is then purified either by gel electrophoresis or by gel filtration chromatography prior to end labeling (see Purification of RNA).

5' End-Labeling⁶

Step 1. Synthesize transcripts *in vitro* without a cap and purify.

Step 2. After the second ethanol precipitation, redissolve 5–10 pmol

⁵ T. Maniatis, E. F. Fritsch, and J. Sambrook, "Molecular Cloning: A Laboratory Manual," p. 401. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982.

⁶ T. Maniatis, E. F. Fritsch, and J. Sambrook, "Molecular Cloning: A Laboratory Manual," p. 122. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982.

- of RNA in 94 μl of water. To remove the 5'-terminal phosphates, add 5 μl of 1 M Tris-HCl, pH 9.0, and 20 U (1 μl) of alkaline phosphatase. Incubate for 1 hr at room temperature.
- Step 3. Remove the alkaline phosphatase with one phenol-chloroform extraction.
- Step 4. Precipitate the RNA with ethanol. Rinse the pellet and redissolve in 7 μl of water. Add 2 μl of $10\times$ T4 polynucleotide kinase buffer, 1 μl (10 U) of T4 polynucleotide kinase, and 10 μl (100 μCi) of [γ - ^{32}P]ATP. Incubate for 1 hr at 37°. Purify the RNA by polyacrylamide gel electrophoresis (PAGE).

3' End-Labeling^{7,8}

3'-End-labeling RNA is discussed in detail in a chapter in a previous volume of this series.⁸

- Step 1. Synthesize transcripts *in vitro* with or without a cap and purify.
- Step 2. After the second ethanol precipitation, redissolve the RNA (at least 5 pmol) in 2 μl of 250 μM ATP, 2 μl of 0.1 mg/ml BSA, 2 μl of $10\times$ T4 RNA ligase buffer, 2 μl of DMSO, and 10 μl (100 μCi) of [5'- ^{32}P]pCp. Cool to 4° before adding 8–20 U (2 μl) of T4 RNA ligase. Incubate at 4° for at least 10 hr. Purify the labeled RNA by gel electrophoresis.

(Note) RNA that ends in a U labels 10-fold less efficiently than RNAs that end in A, C, or G.⁷ We therefore avoid using RNAs with a 3'-terminal U.

Chemical Modification of RNA⁴

Amount of RNA Required

The end-labeling protocols described in End-Labeling Methods yield RNA at a specific activity of approximately 10^6 cpm (counts per minute)/pmol, corresponding to the labeling of 15% of the available termini. The recovery of RNA after the labeling, chemical modification, and aniline cleavage steps is about 80% of the starting material. To sequence a 200-nt (nucleotide) RNA, 3×10^4 cpm/lane is sufficient for an overnight exposure. Therefore, a minimum of 4×10^4 cpm is necessary. This corresponds to 40 fmol of RNA at 10^6 cpm/pmol. This amount of radioactivity must be increased, if losses occur during the reaction of interest.

⁷ T. E. England and O. C. Uhlenbeck, *Biochemistry* **17**, 2069 (1978).

⁸ P. J. Romaniuk and O. C. Uhlenbeck, this series, Vol. 100, p. 52.

Purine Modification (A + G)

This reaction modifies A's more efficiently than G's, in about a 4 : 1 ratio, but can be used to analyze both purines in a single reaction.

- Step 1. Purify the labeled RNA by gel electrophoresis. Use 12.5 μg of yeast RNA as carrier, when eluting the RNA from the gel slice.
- Step 2. Take out the amount needed for the modification. Adjust the amount of yeast RNA in that aliquot to a total of 12.5 μg . Precipitate the RNA with ethanol, rinse the pellet thoroughly with 75% (v/v) ethanol, and dry.
- Step 3. Redissolve the RNA pellet in 200 μl of buffer A. Add 2 μl of fresh DEPC. Vortex for 5 sec and incubate for 2.5 min at 90°. Open the lid on the tube before incubating, otherwise the CO₂ generated will pop the lid open and splash the contents of the tube.
- Step 4. To stop the reaction, add 75 μl of 1 M sodium acetate, pH 4.5, and 750 μl of 100% (v/v) ethanol. Centrifuge for 20 min at 4° to pellet the RNA.
- Step 5. Redissolve the pellet in 200 μl of 0.3 M sodium acetate, pH 3.8. Add 600 μl of ethanol and pellet again. The RNA is now ready to be used as a substrate in the reaction of interest.

Pyrimidine Modification

The pyrimidines can be modified either separately (U only or C only modifications) or together (U + C modification). With 3'-end-labeled RNA, all three types of modifications can be used successfully. With 5'-end-labeled RNA, only the U only modification reliably yields useful data; the U + C and the C only modifications yield unreadable ladders.

U Only Modification

- Step 1. Purify the labeled RNA by gel electrophoresis. Use 12.5 μg of yeast RNA as carrier, when eluting the RNA from the gel slice.
- Step 2. Take out the amount needed for the modification. Adjust the amount of yeast RNA in that aliquot to a total of 12.5 μg . Precipitate the RNA with ethanol, rinse the RNA pellet thoroughly with 75% (v/v) ethanol, and dry.
- Step 3. Redissolve the RNA pellet in 10 μl of water and add 10 μl of anhydrous hydrazine. Hydrazine dissolves readily in water. Incubate for 10 min on ice.

- Step 4. To stop the reaction, add 200 μl of 0.3 M sodium acetate, pH 3.8, and 750 μl of ethanol. Centrifuge for 20 min at 4° to pellet the RNA.
- Step 5. Redissolve in 200 μl of 0.3 M sodium acetate, pH 3.8, add 600 μl of ethanol, and pellet again. The RNA is now ready to be used as a substrate in the reaction of interest.

U + C Modification

This modification should be performed exactly as that in the U only modification described in the previous section, except that, at step 3, the RNA should be redissolved in 20 μl of anhydrous hydrazine–0.5 M NaCl, and the incubation on ice should be for 30 min. The hydrazine–NaCl must be made up fresh, both for this and for the C only modifications described in the following section.

C Only Modification

This modification should be performed exactly as in the U + C reaction described in the previous section, except that, at step 3, the RNA should be redissolved in 20 μl of anhydrous hydrazine–3.0 M NaCl.

Cleavage of Modified RNA with Aniline and Gel Electrophoresis⁴

- Step 1. Separate the products from the unreacted RNA after the reaction of interest. This often can be accomplished by gel electrophoresis. Ethanol precipitate the purified RNA. Wash with 75% (v/v) ethanol and dry.
- Step 2. Redissolve the sample in 20 μl of 1 M aniline. Incubate for 20 min at 60° in the dark.
- Step 3. To stop the reaction and remove the aniline, fill the Eppendorf tube with 1-butanol (about 1.4 ml) and vortex for at least 5 sec. Butanol precipitation is faster than the lyophilization step of Peattie.⁴ Centrifuge for 10 min at room temperature to pellet the RNA. Carefully remove the butanol with a Pasteur pipet. The RNA pellet may not adhere to the side of the tube.
- Step 4. Redissolve the RNA in 150 μl 1% (w/v) SDS.
- Step 5. Fill the tube with butanol again, vortex, and centrifuge for 10 min at room temperature.
- Step 6. Rinse the pellet with 1 ml of 100% (v/v) ethanol. Dry thoroughly. Incomplete drying of the pellet at this point will cause the RNA to smear on the gel.
- Step 7. Redissolve in 5 μl of buffer B. Use of this loading buffer,

rather than a formamide-based loading buffer, results in better resolution on the sequencing gel.

- Step 8. Boil for 90 sec. Chill on ice immediately and load onto a sequencing gel. The sample should not be boiled longer than 90 sec or the urea will crystallize when put on ice. If this happens, centrifuge the tube briefly to bring any condensed water on the sides of the tube to the bottom or add 0.5 μ l of water to the sample.

An aliquot of modified RNA should be reserved as the starting RNA. This sample will yield bands at every modified position and provides a standard to which RNA products can be compared. The comparison is simplest if the same amount of radioactivity is present in the ladders being compared. Thus, before loading the gel, each sample should be counted, and the approximate number of bands expected in each ladder of RNA should be calculated (often this number differs considerably in the RNA substrate and product). Load the same radioactivity per band onto each lane.

Purification of RNA

After both the transcription and the end-labeling reactions, the RNA must be separated from unincorporated nucleotide triphosphates. This can be done either by gel filtration chromatography or by electrophoresis through a denaturing polyacrylamide gel.

*Rapid Column Chromatography*⁹

- Step 1. Remove the cap from a silanized Eppendorf tube and place inside a sterile 17 \times 100-mm tube. Plug a 1-ml disposable syringe with silanized glass wool and place inside the tube.
- Step 2. Fill the syringe with Sephadex G-25 that has been swollen in buffer C. Allow the beads to pack.
- Step 3. Add more Sephadex G-25 until the packed volume is about 1 ml. Discard the excess buffer.
- Step 4. Remove the void volume from the column by centrifugation at 1000 *g* for 5 min at room temperature.
- Step 5. Apply the RNA (100 μ l) to the column. Let the sample equilibrate at room temperature for 5 min.
- Step 6. Recover the sample by centrifugation for exactly the same time at the same temperature as in step 4. The recovered

⁹ T. Maniatis, E. F. Fritsch, and J. Sambrook, "Molecular Cloning: A Laboratory Manual," p. 466. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982.

sample volume should be 95–105 μl . This procedure will remove about 98% of the unincorporated nucleotide triphosphates.

*Polyacrylamide Gel Electrophoresis*⁴

- Step 1. Load the RNA onto a denaturing polyacrylamide gel. A 200-nt RNA can be purified by electrophoresis through a 40-cm-long 6% acrylamide (w/v) sequencing gel at 30 V/cm for 1.5 hr.
- Step 2. Locate the RNA in the gel either by autoradiography or by fluorescence after the gel has been stained with ethidium bromide (EtBr). Cut out the gel slice containing the RNA.
- Step 3. Soak the gel slice in 500 μl of buffer D for at least 2 hr at 37°. If the RNA is unlabeled, 20 μg of glycogen is included as carrier; if the RNA is ³²P labeled, 12.5 μg of yeast RNA is included as carrier.
- Step 4. Transfer the buffer containing the eluted RNA to a new tube and precipitate with ethanol.
- Step 5. Redissolve the RNA in 200 μl of 250 mM NaCl.
- Step 6. Centrifuge for 10 min to remove debris.
- Step 7. Transfer the RNA to a new tube and precipitate with ethanol a second time.
- Step 8. Rinse the pellet in 75% (v/v) ethanol, dry, and redissolve in water, unless otherwise indicated.

Analysis of mRNA 3' End Formation

The following example, an analysis of the requirements for 3' end cleavage of SV40 late precursor mRNA (pre-mRNA), is used to illustrate the method.¹ The details of cleavage and polyadenylation *in vitro* are discussed in detail elsewhere.^{10,11}

In this experiment, we analyzed a reaction in which a pre-mRNA is cleaved at a specific nucleotide [the poly(A) site], producing two "half-molecules."¹ End-labeled RNA modified with either DEPC or hydrazine was incubated in HeLa cell nuclear extract¹² under conditions in which cleavage occurs. RNA was recovered from the extract. The products of the cleavage reaction were separated from the unreacted RNA by gel electrophoresis. These RNAs and the starting RNA were treated with aniline and were analyzed by PAGE.

¹⁰ C. Moore, this series, Vol. 181, p. 6.

¹¹ C. L. Moore and P. A. Sharp, *Cell* **41**, 845 (1985).

¹² J. D. Dignam, R. M. Lebovitz, and R. G. Roeder, *Nucleic Acids Res.* **11**, 1475 (1983).

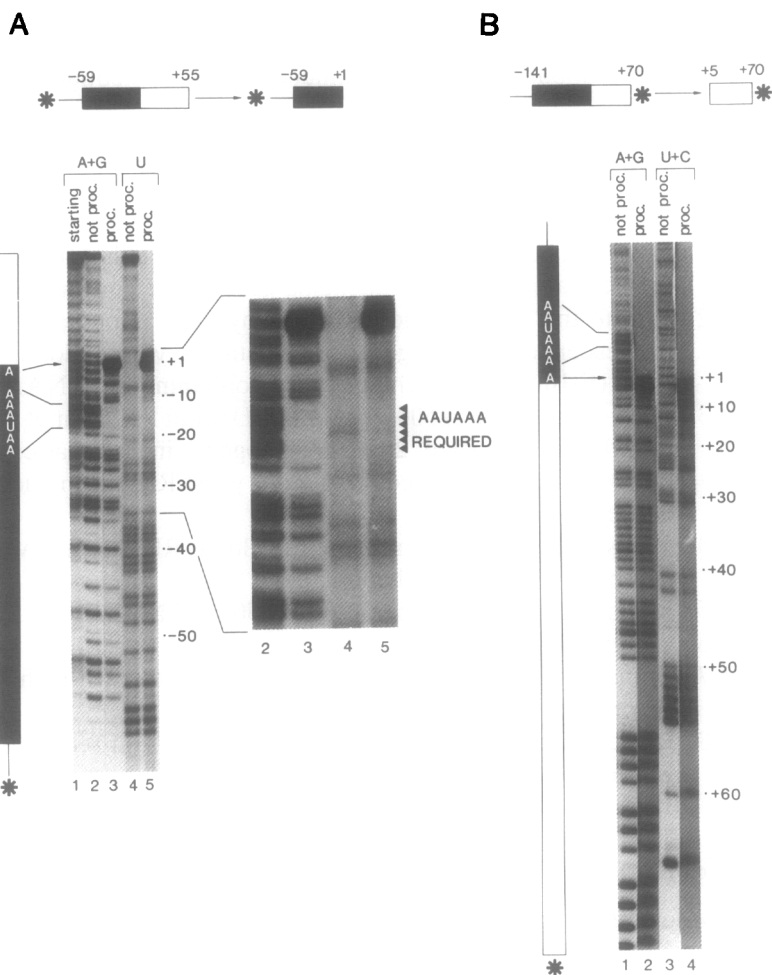


FIG. 3. Nucleotides required for cleavage. (A) Nucleotides upstream of the poly(A) site. 5'-End-labeled $-59/+55$ RNA was treated with DEPC (lanes 1–3) or with hydrazine (lanes 4 and 5) and was incubated in a nuclear extract, containing 0.5 mM EDTA to prevent polyadenylation. 5' Half-molecules were isolated and treated with aniline. (Lane 1) DEPC-treated RNA that was not incubated in the extract. (Lane 2) DEPC-treated RNA that was not cleaved during incubation in the extract. (Lane 3) DEPC-treated RNA that was cleaved in the extract. (Lane 4) Hydrazine-treated RNA that was not cleaved in the extract. (Lane 5) Hydrazine-treated RNA that was cleaved in the extract. The sequence surrounding AAUAAA is enlarged to show detail. Modification of any nucleotide in AAUAAA prevents cleavage in the extract. The decreased intensity of the base at -36 (lane 3) is not reproducible. (B) Nucleotides downstream of the poly(A) site. 3'-End-labeled $-141/+70$ RNA was treated with DEPC (lanes 1 and 2) or with hydrazine (lanes 3 and 4) and was incubated in a nuclear extract, containing 0.5 mM EDTA. 3' Half-molecules were isolated and treated with aniline. (Lane 1) DEPC-treated RNA that was not cleaved in the extract. (Lane 2) DEPC-treated RNA that was cleaved in the extract. (Lane 3) Hydrazine-treated RNA that was not cleaved in the extract. (Lane 4) Hydrazine-treated RNA that was cleaved in the extract. No single nucleotide modification downstream of the poly(A) site prevents cleavage.

The results are shown in Fig. 3. In the analysis of 5'-end-labeled RNA (Fig. 3A), the bases in the hexanucleotide AAUAAA are clearly absent from the processed RNA lanes (compare lanes 2 and 4 to lanes 3 and 5), consistent with results obtained by mutagenesis.¹³ All other bases are present in the processed RNA, both in the 5' half-molecules (Fig. 3A) and in the 3' half-molecules (Fig. 3B). We conclude that the only single modifications that prevent cleavage lie in AAUAAA. We have performed comparable analyses of the formation of processing complexes and the addition of poly(A).¹ Rymond and Rosbash, who developed the modification interference method independently, have applied the method to the splicing of yeast pre-mRNAs.²

Other Modifications

Modification interference requires two steps: modification of specific bases with chemical reagents and the detection of those modified bases in substrate and product RNAs. Reagents other than DEPC and hydrazine satisfy these requirements. For example, dimethyl sulfate (DMS) has been used to examine G, A, and C residues.¹⁴ Other potentially useful reagents include kethoxal (G specific), CMCT¹⁵ (U and G specific),¹⁴ and ethylnitrosourea (ENU) (phosphate specific).¹⁶ Phosphorothioates can be incorporated using phosphorothioate containing nucleotide triphosphates. The modified positions can be identified by sensitivity to aniline cleavage, as is the case with the modifications described in this chapter, or by any other method that provides single-nucleotide resolution. For example, primer extension by reverse transcriptase is blocked by many base modifications and phosphodiester containing a phosphorothioate are readily cleaved with iodoethane.^{14,17}

¹³ M. Wickens and P. Stephenson, *Science* **226**, 1045 (1984).

¹⁴ D. Moazed and H. F. Noller, *Cell (Cambridge, Mass.)* **47**, 985 (1986).

¹⁵ 1-Cyclohexyl-2-morpholinocarbodiimide metho-*p*-toluene sulfonate.

¹⁶ P. Romby, D. Moras, M. Bergdoll, P. Dumas, V. V. Vlassov, E. Westhof, J. P. Ebel, and R. Giege, *J. Mol. Biol.* **184**, 455 (1985).

¹⁷ G. Gish and F. Eckstein, *Science* **240**, 1520 (1988).