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USE OF A 2-5A ANALOGUE PROBE FOR DETECTING RNA LIGASE AND RNA LIGASE SUBSTRATES IN MAMMALIAN CELL EXTRACTS

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The compound ppp(A2'p)$_3$A3'[^{32}P]pCp is a commercially available radioactive analogue of the 2',5' oligoadenylate series ppp(A2'p)$_n$A, n≥2, commonly referred to as 2-5A. It is used as a probe for measuring concentrations in competition radiobinding and radioimmune assays. We have found that incubation of the probe with extracts from HeLa, CV1, or neuroblastoma cells results in its covalent attachment to two size classes of RNA: the first includes a major species with a molecular weight of approximately 350,000, the second is much smaller (40±5 nucleotides in length) and could represent tRNA half-molecules. Ligation is to the 3' end of the probe molecule with formation of a 3',5'-phosphodiester bond. Thus, probe ligation provides a sensitive and convenient assay for the detection not only of RNA ligase(s) but also of ligatable RNAs (such as the putative tRNA half-molecules) in mammalian cell extracts.

The 2',5'-phosphodiester-linked oligoadenylate series ppp(A2'p)$_n$A, n≥2, commonly abbreviated as 2-5A, is synthesized from ATP by an interferon-inducible 2-5A synthetase. The only known function of 2-5A is to activate an endogenous cellular nuclease (the 2-5A-dependent RNase, RNase L or F) which can degrade messenger RNA, produce characteristic cleavages in ribosomal RNA and inhibit protein synthesis. The 2-5A system is activated and may play a part in the antiviral effects of interferon in a number of virus-cell systems. A wider role for 2-5A in mediating the cell growth inhibitory effects of interferon and in systems not involving interferon has also been postulated (for reviews on 2-5A and the 2-5A system see 1-3).

In searching for additional functions for 2-5A in mammalian cells we observed that a radioactive analogue of 2-5A, ppp(A2'p)$_3$A3'[^{32}P]pCp, used as a probe (4) is covalently attached to two distinct size classes of RNA in extracts from HeLa, CV1, and human neuroblastoma cells in an RNA ligase reaction in which the probe provides the 5'-terminal moiety. RNA ligase activity has only recently been demonstrated in mammalian cell-free extracts due, in part, to the difficulty in obtaining suitable substrates for detecting the reaction. Substrates that have been successful are a fragment of tobacco mosaic virus RNA used in a circularization assay (5), and specific precursor tRNA (6) and mRNA (7,8) species used in excision-ligation assays.

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Our fortuitous finding that the 2-5A probe can serve as a substrate for ligation opens up new possibilities for studying both the enzymes and substrates involved in ligation reactions in mammalian cell-free extracts.

MATERIALS AND METHODS

Preparation of cell-free extracts. HeLa cells, CV1 cells, and human neuroblastoma cells were grown to high density as monolayers in 90 mm plastic petri dishes using Dulbecco modified Eagles medium supplemented with 10% heat-inactivated newborn calf serum. The plates were chilled on ice and the medium removed. The cells were rinsed with cold phosphate-buffered saline, scraped into the saline using a rubber policeman, collected by centrifugation and resuspended by vortexing into 2 volumes of cold homogenization buffer (10 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM magnesium acetate, 7 mM 2-mercaptoethanol). The cell suspensions were placed on ice for 1 min and frozen in dry ice. The frozen cell suspensions were lysed by thawing at room temperature with periodic vortex mixing and 1/10 volume of cold 10X buffer (0.1 M HEPES-KOH, pH 7.5, 0.9 M KCl, 0.015 M magnesium acetate, 0.07 M 2-mercaptoethanol) added. The cell extracts were mixed, centrifuged at 10,000 X g for 15 min, and the supernatant fractions (S10s) removed and frozen in aliquots at -70°C. Where indicated post-ribosomal supernatant fractions (S100s) were obtained by centrifugation of the S10s for 30 min in a Beckman Airfuge.

Ligase reaction conditions. Ligase reactions were carried out for 45 min at 30°C, or, where indicated, at 0 or 20°C, in microcentrifuge tubes with 5 µl cell extract (S10), 15 µl H_2O (this dilution of the extract was essential for good ligase activity) and 1 µl of probe ($ppp(A2'p)_3A3'[^{32}P]pCp$, Amersham International; 2-3 X 10^6 Ci/mol; 0.1 mCi/ml). Where higher concentrations of probe were added (Fig. 1, lanes 4-7), methanol and ammonium phosphate were removed by passage through a 1 ml Sephadex G25 column equilibrated with distilled H_2O. The probe-containing excluded fractions were combined, lyophilized almost to dryness, and reconstituted with H_2O to a concentration corresponding to that of the original probe. When $[5'-^{32}P]pCp$ (Amersham International; 2-3 X 10^6 Ci/mol) was substituted for the 2-5A probe (Fig. 1; lane 13) 0.25 µCi was added per reaction.

Analysis by SDS-polyacrylamide gel electrophoresis. Ligase reactions were mixed with an equal volume of 2X sample buffer (40% glycerol, 4% SDS, 0.08 M Tris-HCl, pH 6.8, 0.14 M 2-mercaptoethanol), heated at 90°C for 5 min and electrophoresed on 15% SDS-polyacrylamide slab gels for 3 h at 150 volts (9). The gels were dried and the radioactive bands located by autoradiography at -70°C for 24 h using Fuji RX X-ray film and a Kodak intensifying screen. Where indicated RNAs in the reaction mixtures were isolated by phenol extraction and the alcohol-precipited RNA dissolved in 1X sample buffer and analyzed by electrophoresis as above.

Isolation of X and Z. RNA was isolated from a large scale (800 µl) ligase reaction mixture by phenol extraction and ethanol precipitation. This precipitate was dissolved in 0.01 M Tris-HCl (pH 7.5), 1 mM EDTA and the RNA separated from unreacted probe by passage through a Sephadex G50 fine column in the same buffer. The gel-excluded peak of radioactivity contained X, Y and Z which were separated by further chromatography through a Sephadex G100 superfine column. The excluded fractions contained X as the only radioactive species whereas the late-appearing fractions contained only Z.

Ribonuclease digestions. All ribonucleases were obtained from Sigma Chemical Co. Samples (10 µl) of X and Z (adjusted to pH 4.5 with 0.5 µl of 1 M ammonium acetate for T2 RNase) were incubated at 37°C with 1 µl of pancreatic (100 µg/ml), T2 (100 U/ml), or T1 (2,000 U/ml) RNase for 45, 45 or 30 min, respectively. RNA concentrations were 600 µg/ml in X and 50 µg/ml in Z.

RESULTS

Nature of the reaction. Incubation of diluted HeLa cell extracts with the 2-5A probe $[ppp(A2'p)_3A3'[^{32}P]pCp]$ at 30°C (Fig. 1, lane 3) produced three major

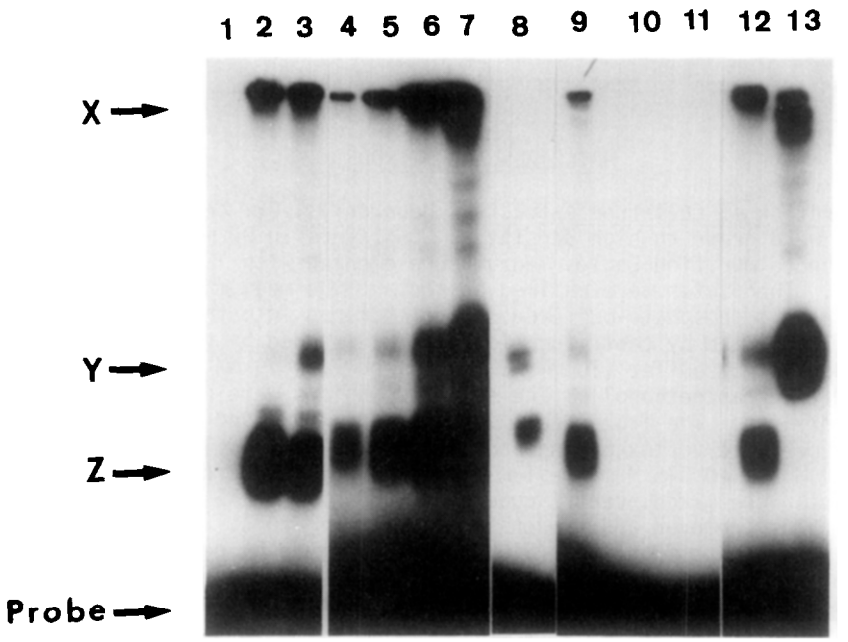

Fig. 1. Characteristics of the ligase reaction. SDS-polyacrylamide gel electrophoresis of reactions incubated with HeLa cell extract (S10) for 45 min. Lanes 1-3, incubations were at 0°C, 20°C, and 30°C respectively. Lanes 4-7, 0.07, 0.2, 0.7, and 2.0 μCi respectively of probe. Lane 8, reaction with a post-ribosomal supernatant fraction (S100) from HeLa cells. Lanes 9-11, nucleic acid isolated from a reaction mixture by phenol extraction and ethanol precipitation without (lane 9) and after digestion with pancreatic (lane 10) or T2 (lane 11) RNase. Lanes 12 and 13, 0.2 μCi probe and 0.25 μCi[5'-^{32}P]pCp respectively.

size classes of radioactive products designated X, Y and Z resolvable by electrophoresis on SDS-polyacrylamide gels (radioactivity at the bottom of the gel corresponds to unreacted probe). At 0°C (lane 1), little or no X, Y and Z were formed, suggesting that these products are the result of enzymic reactions and not the adventitious binding of probe to cellular material. Incubation at 20°C (lane 2) significantly reduced incorporation into Y without affecting X and Z. Product Y has a molecular weight of approximately 25,000 and is thought to reflect degradation of probe and re-incorporation of liberated [5'-^{32}P]pC into the -CCA termini of tRNAs (see below). The amount of probe routinely employed (Materials and Methods) is limiting; the addition of more resulted in the formation of proportionally more X, Y and Z as well as a number of minor products (lanes 4-7). Incubation with a post-ribosomal supernatant fraction (S100) from HeLa cells resulted in the labelling of Y and Z only (lane 8), indicating that X is ribosome-derived.

Reaction mixtures boiled in 0.1% SDS containing 10 μM nonradioactive 2-5A, phenol extracted, ethanol precipitated and analyzed by SDS polyacrylamide gel electrophoresis yielded the same products X, Y and Z (Fig. 1, lane 9) which were completely destroyed by digestion with pancreatic RNase (lane 10) or T2 RNase (lane

11). In contrast, treatment of the original incubation mixture with proteinase K eliminated all protein-staining bands on the gel but had no effect on X, Y and Z (gel not shown).

Incubation with [5'-^{32}P]pCp yielded radioactive products which comigrated with X and Y but not Z (compare lanes 13 and 7, Fig. 1). With probe, 100 μM CTP completely inhibited the labelling of Y but had no effect on X or Z (not shown); with [5'-^{32}P]pCp it similarly inhibited Y but only partially inhibited "X". These results are consistent with a ligase reaction(s) for X and Z but some probe breakdown and the labelling of Y by the incorporation of radioactive CTP into the CCA terminus of tRNA. They also suggest that the ligase responsible for the formation of X (but not Z) can utilize pCp. The partial inhibition of incorporation of the latter by CTP, however, emphasizes its unsuitability as an alternative probe. Incubation of extracts from both monkey CV1 cells and human neuroblastoma cells with probe also resulted in the formation of both X and Z (results not shown).

Size analysis of the RNA products. Sephadex-purified X (Methods) was denatured and subjected to agarose gel electrophoresis (Fig. 2A, lane 3) in parallel with RNA from an S10 incubated ± 2-5A (lanes 1 and 2). For the latter the arrows indicate the positions of the ethidium bromide stained 18 and 28S rRNAs and the major 2-5A mediated human rRNA cleavage products a and b (10). Radioactive X (lane 3) migrated slightly slower than b (visible on the dried ethidium bromide stained gel used for autoradiography) at a position indicating a molecular weight of 350,000. Other preparations of X frequently contained varying amounts of additional products that produced a low intensity smear of radioactivity in the same region of the gel (cf Fig. 1, lane 7). On electrophoresis through a sequencing gel Z migrated to a position corresponding to a length of 40±5 nucleotides (Fig. 2B, lane 3).

Nature of the probe-RNA linkage. The nature of the linkage of probe to X and Z was investigated by thin layer chromatography (TLC) analysis of their RNase digestion products (Fig. 3). Probe as supplied by Amersham International consists of a major species of ppp(A2')$_3$A3'[^{32}P]pCp and a minor species of the corresponding 5'-diphosphate (Fig. 3, lane 1). The predominantly 2',5'-linked probe is resistant to digestion with pancreatic RNase and on T2 RNase digestion yields (p)pp(A2'p)$_3$A3'[^{32}P]p which is indistinguishable from probe in this TLC system (lanes 1 and 4). Purified X does not move from the origin during chromatography (lane 5) but digestion with pancreatic RNase (lane 7) and T2 RNase (lane 8) generates oligonucleotides migrating the same as probe, whereas partial digestion with T1 RNase (lane 6) yields products intermediate in size between probe and starting material. Similar results were obtained with Z (lane 9) hydrolyzed with pancreatic RNase (lane 10), T2 RNase (lane 11) and T1 RNase (lane 12) (see also lanes 1 and 2, Fig. 2B). These results were corroborated by analyzing the products from T2 and pancreatic RNase digestion of X and Z by high performance liquid chromatography (11). Digestion of either with T2 RNase produced (p)pp(A2'p)$_3$A3'[^{32}P]p whereas hydrolysis with pancreatic RNase liberated the original

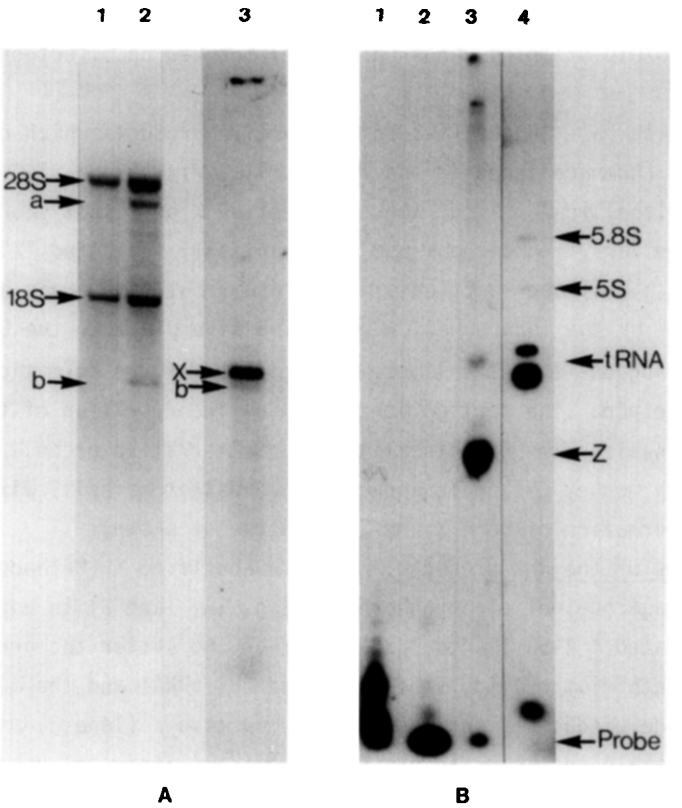

Fig. 2. Size analysis of ligase reaction products X and Z. Panel A. Radioactive X (lane 3) was denatured and analyzed by electrophoresis on a 1.8% agarose gel (10) in parallel with RNA from a HeLa S10 incubated at 30°C for 60 min minus (lane 1) or plus (lane 2) 100 nM 2-5A. An autoradiograph (lane 3) and a photograph (lanes 1 & 2) of the ethidium bromide stained gel are presented. The 18 and 28S rRNAs and the major 2-5A-mediated rRNA cleavage products a and b are arrowed to the left. The position of b in lane 3 was determined from the dried stained gel used for autoradiography. Panel B. Electrophoretic analysis of Z in an 8% acrylamide sequencing gel in the presence of urea, without (lane 3) or after partial or total digestion (Methods) with T1 (lane 1) or pancreatic (lane 2) RNase respectively. Lane 4, marker RNAs extracted from a HeLa S10 and labelled with [^{32}P]pCp using T4 ligase (12).

probe (p)pp(A2'p)$_3$A3'[^{32}P]pCp rather than (p)pp(A2'p)$_3$A3'[^{32}P]pC2'pNp, thus excluding the formation of a 2',5' linkage in the ligase reaction (data not shown).

Structural requirements of probe for ligation. Experiments utilizing probe analogues without 5'-terminal phosphates (Fig. 4, lanes 1-4) and containing adenylate residues linked through either 3',5'-(lanes 1 and 2) or 2',5'-(lanes 3 and 4) phosphodiester bonds showed that neither 5'- di- or tri- phosphates nor 2',5'-phosphodiester bonds are required for ligation. However, removal of the 3'-terminal phosphate (lanes 6 and 7) or the 3' terminal Cp (lanes 9 and 10) destroyed the ability of these analogues to serve as substrates in the reaction.

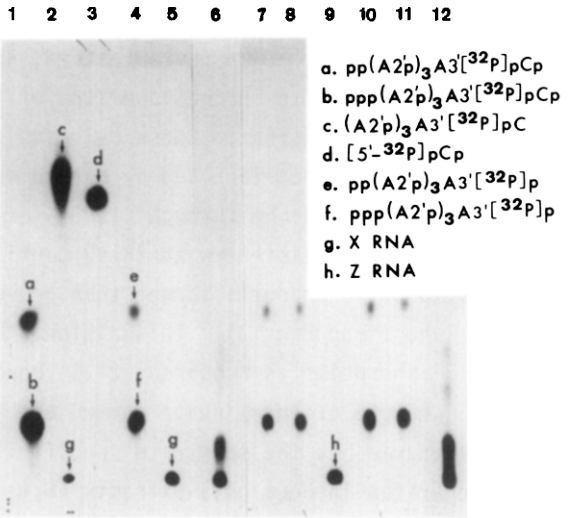

Fig. 3. Thin-layer chromatographic analysis of RNase digestion products of X and Z. TLC was performed using thin layer plates of poly (ethyleneimine)-cellulose (Camlab, Cambridge, England). These were run (ascending) for 3 h in 0.75 M KH_2PO_4, pH 3.4. Lane 1, probe; lane 2, probe with terminal phosphates removed (top) together with purified X (bottom); lane 3, [5'-^{32}p]pCp marker; lane 4, probe with terminal-Cp removed by digestion with T2 RNase; lane 5, purified X; lane 6, X partially digested with T1 RNase; lanes 7 and 8, X digested with pancreatic or T2 RNase respectively; lane 9, purified Z; lane 10 and 11, Z digested with pancreatic or T2 RNase respectively; lane 12, Z partially digested with T1 RNase.

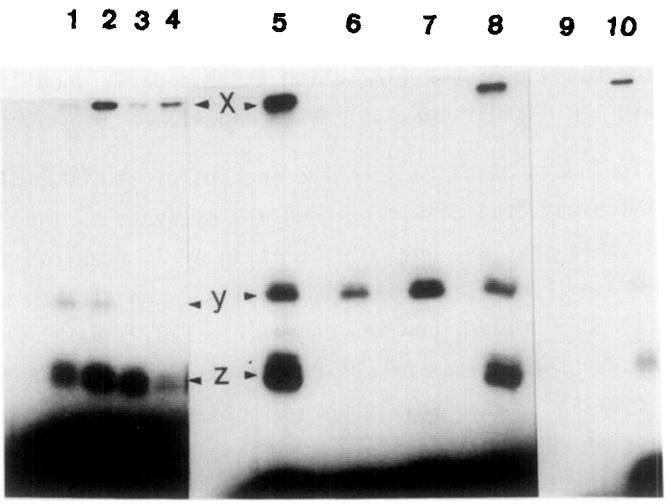

Fig. 4. Ligation reactions using modified probes. All reactions were carried out with HeLa cell extracts at either 20°C (lanes 1-4) or 30°C (lanes 5-10) and using approximately 0.2 μCi (4 nM) of probe or probe analogue per reaction. Ligations were with: probe alone (lane 5); (A3'p)$_3$A3'[^{32}P]pCp (lanes 1 and 2) or (A2'p)$_3$A3'[^{32}P]pCp (lanes 3 & 4) in extracts preincubated (lanes 2 & 4) or not (lanes 1 & 3) with 100 μM 2-5A for 15 min at 30°C before addition of the radioactive analogue; (A2'p)$_3$A3'[^{32}P]pC in extracts preincubated (lane 7) or not (lane 6) with 2-5A as above; probe in the presence of (A2'p)$_3$A3'[^{32}P]pC (lane 8); ppp(A2'p)$_3$A3'[^{32}P]p in the absence (lane 9) or presence (lane 10) of probe.

DISCUSSION

The incubation of extracts from HeLa, human neuroblastoma, and monkey CV1 cells with a (p)pp(A2'p)$_3$A3'[^{32}P]pCp probe results in the labelling of two size classes of RNA, X and Z. Digestion of these with pancreatic RNase releases the probe intact (Fig. 3), demonstrating that X and Z are not labelled by breakdown and de novo synthesis but rather by the direct ligation of probe through its 3'-terminal phosphate to the 5' end of acceptor X and Z RNAs. Consistent with this, partial hydrolysis of X and Z with T1 RNase yielded radioactive products larger than probe but considerably smaller than X or Z (Fig. 2B, lane 1 and Fig. 3). In addition, the structural requirements for probe to react (the 3' phosphate is required, 2',5' bonds or 5' triphosphate are not required) and the 3',5' nature of the linkage formed are in accord with a ligation reaction in which the enzyme has the substrate specificity of the ligase recently identified and characterized in HeLa cell extracts (5,6). This ligase couples through a 3',5'-phosphodiester bond an RNA moiety with a 3'-terminal phosphate to an acceptor species bearing a free 5'-hydroxyl group.

The ligase specificity means that the 2-5A probe is a 3'-terminal phosphate-bearing substrate for ligation. The facts that it is commercially available and that it is much more resistant than 3',5'-phosphodiester-linked nucleotides to nuclease degradation make it an attractive reagent for studying RNA ligation in vitro. It has the further advantage that it can detect both ligase activity and ligatable RNA species in extracts. In searching for the latter it should be remembered that ligatable RNAs will be generated through the cleavage of rRNA and mRNA by probe-activated 2-5A-dependent RNase in the extracts. This complication can be overcome by use of the 2-5A analogue (A2'p)$_3$A3'[^{32}P]pCp (12) which is an equally good ligase substrate but does not activate the RNase (Fig. 4, lanes 3 and 4).

Region X RNAs include a major radioactive species of 350,000 molecular weight (Fig. 2A, lane 3) which may originate from rRNA via cleavage by the 2-5A-dependent RNase. Formation of this species, but not formation of Z, requires activation of the 2-5A-dependent RNase (Fig. 4, lanes 1-4). Region Z contains an unusual species of radioactive RNA(s) 40±5 nucleotides in length (Fig. 2B, lane 3) which is considerably smaller than any of the class of Ly1-Ly5 small cytoplasmic RNAs or U1-U6 small nuclear RNAs (13-15). Based solely upon its size we postulate that Z RNA may represent a class of tRNA half-molecules. The generation of the latter and their subsequent ligation to form mature tRNA species has been demonstrated with yeast tRNA precursors in extracts from yeast (16,17), wheat germ (18), and HeLa cells (6), and with a *Xenopus laevis* tRNA^{Tyr} gene transcript in HeLa cell extracts (19), but there is little direct evidence that tRNA half-molecules occur naturally in mammalian cells. Newly synthesized tRNA half-molecules size RNA has been reported in isolated L cell nuclei (20) and hybridization experiments suggest that this RNA may contain tRNA^{Tyr} sequences (21). The selective labelling of Z

with probe in primate cell extracts should make it possible to isolate Z RNA and examine critically its relationship to tRNA.

REFERENCES

1. Lengyel, P. (1982) Ann. Rev. Biochem. 51,251-282.
2. Sen, G.C. (1982) Prog. Nucl. Acids Res. Mol. Biol. 27,105-156.
3. McMahon, M., and Kerr, I.M. (1983) Soc. of Gen. Microbiology Symp. In "The Interferons" D.C. Burke, and A.J. Morris, eds. pp. 89-108.
4. Knight, M., Cayley, P.J., Silverman, R.H., Wreschner, D.H., Gilbert, C.S. Brown, R.E., and Kerr, I.M. (1980) Nature 288,189-192.
5. Filipowicz, W., Konarska, M., Gross, H.J. and Shatkin, A.J. (1983) Nucleic Acids Res. 11,1405-1418.
6. Filipowicz, W., and Shatkin, A.J. (1983) Cell 32,547-557.
7. Hernandez, N., and Keller, W. (1983) Cell 35,89-99.
8. Padgett, R.A., Hardy, S.F., and Sharp, P.A. (1983) Proc. Natl. Acad. Sci. USA 80,5230-5234.
9. Laemmli, U.K. (1970) Nature 227,680-685.
10. Silverman, R.H., Cayley, P.J., Knight, M., Gilbert, C.S., and Kerr, I.M. (1982) Eur. J. Biochem. 124,131-138.
11. Cayley, P.J., Brown, R.E., and Kerr, I.M. (1982) J. Liquid Chromatography 5,2027-2-30.
12. Silverman, R.H., Wreschner, D.H., Gilbert, C.S., and Kerr, I.M. (1981) Eur. J. Biochem. 115,79-85.
13. Zieve, G.W. (1981) Cell 25,296-297.
14. Lerner, M.R., and Steitz, J.A. (1981) Cell 25,298-300.
15. Busch, H., Reddy, R., Rothblum, L., and Choi, Y.C. (1982) Ann. Rev. Biochem. 51,617-654.
16. Greer, C.L., Peebles, C.L., Gegenheimer, P., and Abelson, J. (1983) Cell 32, 537-546.
17. Peebles, C.L., Gegenheimer, P., and Abelson, J. (1983) Cell 32,525-536.
18. Schwarz, R.C., Greer, C.L., Gegenheimer, P., and Abelson, J. (1983) J. Biol. Chem. 258,8374-8383.
19. Laski, F.A., Fire, A.Z., RajBhandary, U.L., and Sharp, P.A. (1983) J. Biol. Chem. 258,11974-11980.
20. Winicov, I., and Button, J.D. (1982) Mol. Cell. Biol. 2,241-249.
21. Winicov, I. (1983) Abstracts of papers presented at the Meeting on RNA Processing, Cold Spring Harbor Laboratory, p. 97.