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U4 and U6 RNAs coexist in a single small nuclear ribonucleoprotein particle

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ABSTRACT

U4 and U6 RNAs of mammalian cells possess extensive intermolecular sequence complementarity and hence have the potential to base pair. A U4/U6 RNA complex, detectible in nondenaturing polyacrylamide gels, is released when human small nuclear ribonucleoproteins (snRNPs) containing U1, U2, U4, U5, and U6 RNAs are dissociated with proteinase K in the presence of sodium dodecyl sulfate. The released RNA/RNA complex dissociates with increasing temperature, consistent with the existence of specific base-pairing between the two RNAs. Since U6 RNA is selectively released from intact snRNPs under the same conditions required to dissociate the U4/U6 RNA complex, the RNA-RNA interaction may be sufficient to maintain U4 and U6 RNAs in the same snRNP particle. The biological implications of these findings are discussed.

INTRODUCTION

Small nuclear ribonucleoproteins (snRNPs) found in the cells of higher eukaryotic organisms are reactive with antibodies from patients with autoimmune diseases (1,2). Anti-Sm antibodies recognize snRNPs containing U1, U2, U4, U5, and U6 RNAs, whereas anti-(U1)RNP antibodies react specifically with snRNPs containing only U1 RNA (1). Evidence from in vitro experiments (3,4,5) supports the hypothesis that U1 snRNPs participate in the splicing of intervening sequences from messenger RNA precursors (2,6). Although analogous roles in RNA processing or biogenesis have been suggested for the other related snRNPs, such proposals have not been experimentally verified.

The nucleotide sequences of the five U RNAs contained in Sm snRNPs are highly conserved across metazoan species (7-15). The protein components of mammalian snRNPs have recently been characterized (16-21). At least nine distinct proteins of molecular weights 11,000 to 68,000 are associated with biochemically purified U1 snRNPs (16,17); three of these carry antigenic determinants recognized by anti-(U1)RNP antibodies (18) and hence are unique to U1 snRNPs. Three of the remaining proteins react with anti-Sm antibodies (18); thus one or more of these proteins must be common to all Sm snRNPs. The discovery of anti-(U2)RNP antibodies has allowed the identification of a

distinct snRNP particle containing U2 RNA and six different proteins, at least one of which is unique to U2 snRNPs (19). The protein compositions of snRNPs containing U4, U5, and U6 RNAs are the least well-defined, since snRNPs containing only one of these RNAs have yet to be isolated by either biochemical or immunochemical means.

Recently, Bringmann et al. presented strong evidence that U6 RNA interacts with some other Sm snRNP particle (22). They observed that anti-$m^{2,2,7}G$ antibodies precipitate deproteinized rat U1, U2, U4, and U5 RNAs, whereas U6 RNA, which does not possess a 5' cap structure containing $m^{2,2,7}G$, is not precipitated. However, if intact snRNPs contained in cell extracts are reacted with the antibodies, U6 RNA is coprecipitated.

Here we report that the particle with which U6 RNA interacts is the U4 snRNP. This association raises intriguing questions concerning the function of these two RNAs in nuclear metabolism.

EXPERIMENTAL PROCEDURES

Computer analysis

Complementarity between U4 and U6 RNA sequences was identified by using the OVRLP program described by Staden (23).

Cells, sera, cell extracts, RNAs

Hela cells were maintained and labeled at 2 X 10^5 cells/ml with [^{32}P] orthophosphate (Amersham) for 12-13 h as described previously (24). Sera from patients with systemic lupus erythematosus were provided by Dr. John Hardin (Yale University). Patient sera (24) and whole cell extracts (25) were prepared as described previously. Unlabeled or in vivo ^{32}P-labeled RNAs were isolated from either a nuclear extract (1) or an anti-Sm precipitate (see below) of a whole cell extract. RNAs were fractionated in a 10% polyacrylamide 7M urea gel (25) and the RNAs in individual bands eluted from gel slices by the crush and soak method (26).

Immunoprecipitation of Sm snRNPs from radiolabeled cell extracts

A procedure based on that of Matter et al. (27) was used. 10 µl of anti-Sm serum was incubated with 2.5 mg protein A Sepharose (Pharmacia) in 500 µl NET-2 (150 mM sodium chloride, 50 mM Tris-HCl pH 7.4, 0.05% Nonidet P-40) for 2 h at room temperature and then washed three times with 500 µl IPP (500 mM sodium chloride, 10 mM Tris-HCl pH 8.0, 0.1% Nonidet P-40). The antibody-bound beads were incubated with 200 µl of extract from 2 X 10^6 cells and 300 µl NET-2 for 1 h at 4°C and then washed three times with 500 µl IPP.

Proteinase K digestion of immunoprecipitated Sm snRNPs

Immunoprecipitated snRNPs were resuspended in 20 or 40 µl proteinase K (EM Biochemicals)/sodium dodecyl sulfate (SDS) solution (2 mg/ml proteinase K, 2% SDS, 150 mM sodium chloride, 40 mM Tris-HCl pH 7.4) and incubated for 1 h at 20°C with occasional shaking. After the Sepharose beads were pelleted by centrifugation for 15 sec in an Eppendorf centrifuge, 20 µl of supernatant was removed and mixed with 4 µl of 30% glycerol containing xylene cyanole FF and bromophenol blue in water. Samples were loaded onto a 10% acrylamide/0.38% bisacrylamide gel (16 X 16 X 0.5 cm) in 40 mM Tris-acetate pH 8.0, 5 mM magnesium acetate (28) and electrophoresed at 4°C and 150 V until the xylene cyanol dye reached the bottom of the gel. The electrophoresis buffer was recirculated between the top and bottom troughs. Gels were usually autoradiographed wet at room temperature.

Melting of the U4/U6 RNA complex

Immunoprecipitated snRNPs from 8 X 10^6 ^{32}P-labeled cells were resuspended in 160 µl of proteinase K/SDS solution and incubated at 20°C for 1 h. After the Sepharose beads were pelleted, the supernatant was divided into seven 20 µl samples. Each sample was mixed with 1 µl 100 mM phenylmethylsulfonyl fluoride (Sigma) in isopropanol to prevent further proteinase K digestion, incubated for 3 min at a temperature indicated in Figure 2A, and chilled on ice. Samples were analyzed as described above.

Release of U6 RNA from immunoprecipitated Sm snRNPs

Immunoprecipitated snRNPs from 2 X 10^6 ^{32}P-labeled cells were resuspended in 400 µl TBS (150 mM sodium chloride, 40 mM Tris-HCl pH 7.4), incubated for 10 min at a temperature indicated in Figure 4, then chilled on ice for 5 min. After centrifugation for 5 sec in an Eppendorf centrifuge, 400 µl of supernatant was removed and RNA was extracted with phenol/chloroform/isoamyl alcohol (50/50/1) containing 0.1% hydroxyquinoline and precipitated with ethanol in the presence of 14 µg/ml carrier RNA. After the pellet was washed once with 500 µl IPP and resuspended in 400 µl IPP containing 1% SDS, RNA was extracted and precipitated as described above. Samples were analyzed in a 10% acrylamide/0.38% bisacrylamide gel (20 X 20 X 0.5 cm) containing 7 M urea in 100 mM Tris-borate, 2 mM Na_2EDTA, pH 8.3 (25).

RESULTS

Sequence complementarity between U4 and U6 RNAs

Mammalian U4 and U6 RNAs exhibit remarkable sequence complementarity.

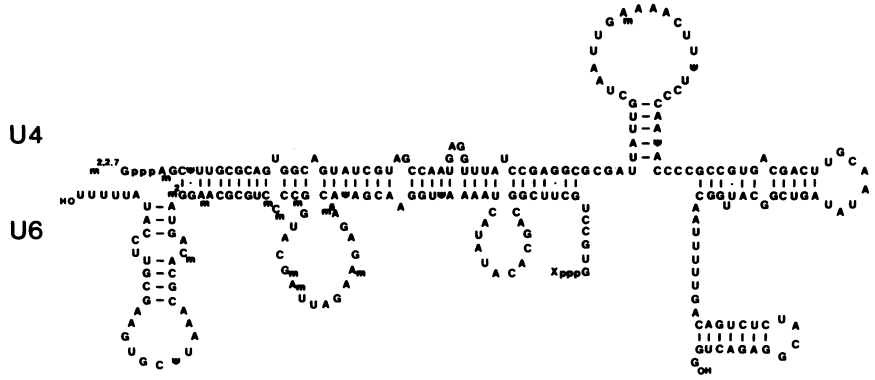

Figure 1. Hypothetical structure showing maximum base-pairing between U4 and U6 RNAs. The sequences are those of rat U4 (11) and U6 (14) RNAs. Human U4 RNA (11) differs from rat U4 RNA by only one nucleotide; human U6 RNA has not been sequenced, but its fingerprint (J. Rinke and J. Steitz, unpublished data) is consistent with the rat U6 sequence (14). Mouse U6 (13) differs from rat U6 in only two positions, which would not alter the interaction shown. The secondary structure in the region of U4 not base-paired to U6 is from reference 11.

Several base-pairing schemes are possible, but the structure shown in Figure 1 displays the most extensive, though interrupted, double-helix formation between the two RNAs. To determine if such base-pairing between U4 and U6 RNAs actually exists in snRNP particles, we performed the following experiments.

A U4/U6 RNA complex is released upon disassemby of snRNPs

SnRNPs immunoprecipitated with anti-Sm antibodies from an extract of ^{32}P-labeled Hela cells were digested with proteinase K in the presence of SDS and the disassembled snRNPs subjected to electrophoresis in a nondenaturing polyacrylamide gel (see Experimental Procedures). Figure 2A (lane 1) shows the profile of RNAs released by this treatment. Each labeled band was excised from the gel and its component RNA(s) identified by their mobility in a well-characterized denaturing polyacrylamide gel system (Fig. 2B). Only the band labeled U4/U6, when analyzed in this manner, was found to contain predominantly two RNAs, U4 and U6 (Fig. 2B, lane 2). All other bands contained predominantly one U RNA (lanes 3-7) which migrated in the nondenaturing gel at a position consistent with its size.

If the gel band containing U4 and U6 RNAs represents a specific base-paired complex, then the hybrid should exhibit the melting behavior expected of an RNA duplex. Melting studies were therefore performed on snRNPs disas-

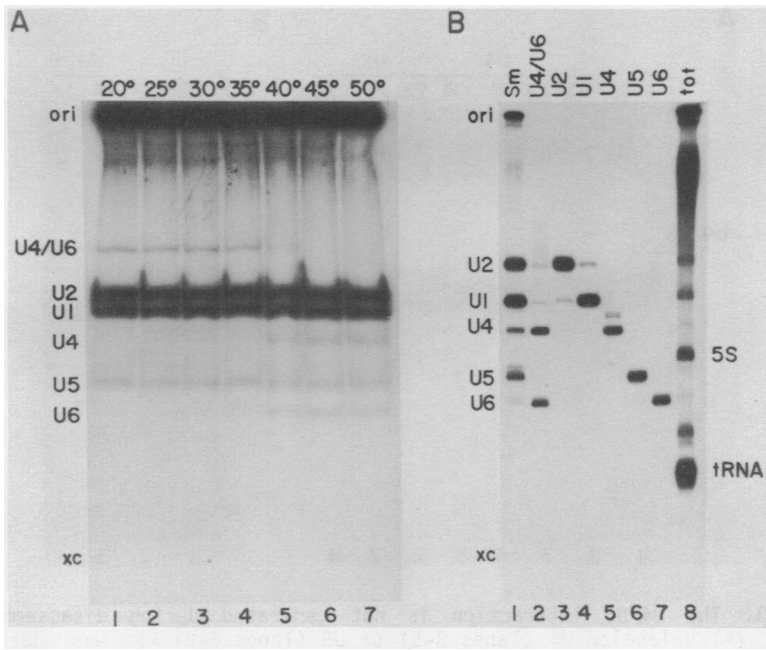

Figure 2. (A) Release and dissociation of the U4/U6 RNA complex from disassembled snRNPs. Immunoprecipitated snRNPs were disassembled by exposure to proteinase K and SDS and then incubated for 3 min at the temperatures (°C) indicated prior to electrophoresis in a nondenaturing 10% polyacrylamide gel (see Experimental Procedures). (B) Each of the labeled bands from lanes 1 and 7 of (A) was eluted from the gel and analyzed in a denaturing 10% polyacrylamide 7M urea gel using as markers the U RNAs in an anti-Sm precipitate (lane 1) and total RNAs (lane 8) from an in vivo ^{32}P-labeled Hela cell extract.

sembled with proteinase K and SDS as described above. After phenylmethylsulfonyl fluoride was added to prevent further proteinase digestion (data not shown), the samples in 150 mM sodium chloride, pH 7.4, were incubated at various temperatures for 3 min prior to electrophoresis in the nondenaturing polyacrylamide gel. Figure 2A shows that the U4/U6 band begins to disappear at 35°C (lane 4), indicating dissociation of the complex; complete dissociation is observed after exposure to 45°C (lane 6). Densitometry of the gel indicates that the approximate Tm (midpoint of the transition from hybrid to dissociated RNAs) is 37°C. The sharpness of the observed dissociation suggests that the interaction between the two RNAs involves specific base-pairing.

The results of several control experiments argue that the U4/U6 interaction we detect is not generated in vitro during the deproteinization step.

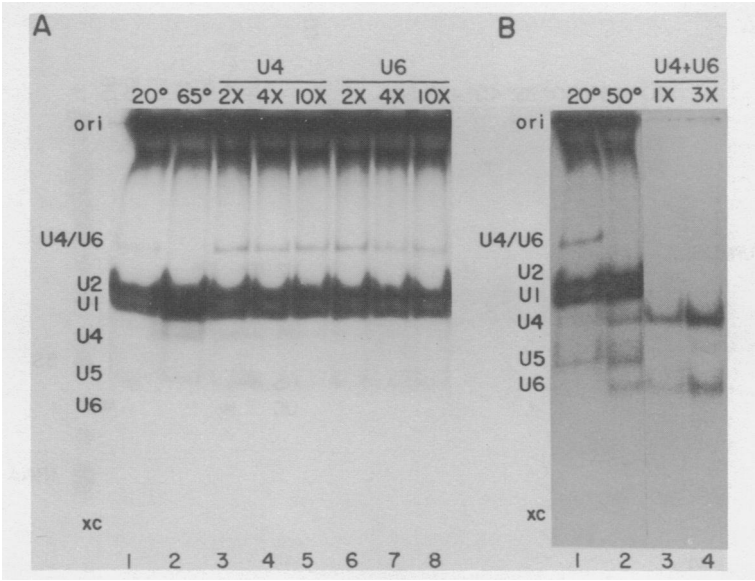

Figure 3. The U4/U6 interaction is not generated during disassembly of snRNPs. (A) Unlabeled U4 (lanes 3-5) or U6 (lanes 6-8) RNA was added to a proteinase K digestion of *in vivo* ^{32}P-labeled snRNPs at 2, 4, or 10 times the amount of these RNAs *in the* immunoprecipitated snRNPs. A proteinase K digest of the same amount of snRNPs as used in lanes 3-8 was incubated at 20°C (lane 1) or 65°C (lane 2) prior to electrophoresis in a nondenaturing 10% polyacrylamide gel. (B) *In vivo* ^{32}P-labeled isolated U4 and U6 RNAs were added to a proteinase K digestion of unlabeled snRNPs at 1 or 3 times (lanes 3 and 4) the amount of these RNAs in the immunoprecipitated snRNPs. A proteinase K digest of *in vivo* ^{32}P-labeled snRNPs was incubated at 20°C (lane 1) or 50°C (lane 2) prior to electrophoresis in a nondenaturing 10% polyacrylamide gel. The snRNPs used in lanes 1 and 2 were prepared from a different *in vivo* ^{32}P-labeled Hela cell extract than was used to prepare the labeled RNAs in lanes 3 and 4.

First, if U4 and U6 RNAs associate only after they are freed of snRNP proteins, then it should be possible to inhibit the appearance of the ^{32}P-labeled U4/U6 band by the addition of competing amounts of unlabeled U4 or U6 RNA to the reaction. Figure 3A shows that the presence of unlabeled U4 RNA (lanes 3-5) or U6 RNA (lanes 6-8), even at concentrations up to ten times the amount of labeled U4 or U6 RNA present in the immunoprecipitated snRNPs, does not diminish significantly the intensity of the U4/U6 band. Second, addition of ^{32}P-labeled U4 and U6 RNAs to a disassembly reaction containing unlabeled snRNPs does not result in significant association of the two labeled RNAs (Fig. 3B, lanes 3 and 4). Finally, we have not yet been successful in

efficiently producing the U4/U6 RNA complex starting with isolated molecules of U4 and U6 RNAs. Incubation of the two RNAs at 20°, 37°, or 50°C for 7 to 30 min in 150 or 500 mM sodium chloride, pH 7.5, with or without Mg^{++}, resulted in at most about 5% association. In these trials, the concentration of each RNA was approximately equal to that in a snRNP disassembly reaction, so it is possible that more efficient annealing could be obtained with higher concentrations of RNAs.

Release of U6 RNA from intact snRNP particles

To ask whether base-pairing might be the major force maintaining U4 and U6 RNAs in a single snRNP particle, we performed melting experiments directly on immunoprecipitated snRNPs. SnRNPs bound to protein A Sepharose beads via anti-Sm antibodies were resuspended in the same buffer (minus the proteinase K and SDS) used in the experiments of Figures 2A and 3 and then incubated for 10 min at various temperatures. After incubation, snRNPs were pelleted and the RNAs present in both the supernatants and pellets were analyzed in a denaturing gel (see Experimental Procedures). Figure 4 (lanes 2 and 5) shows that incubation at 37°C releases into the supernatant about 50% of the U6 RNA present in precipitated snRNPs maintained at 0°C (lanes 1 and 4); incubation at 50°C releases all the U6 RNA (lanes 3 and 6). In contrast, the majority of each of the other U RNAs remains in antibody-bound snRNP particles even after exposure to 50°C. Since 37°C is also the approximate Tm of the deproteinized U4/U6 RNA complex, this experiment suggests that U6 RNA is either not at all or very weakly bound by Sm antigenic protein(s). Moreover, base-pairing could be sufficient to maintain U4 and U6 RNAs in the same snRNP particle. We cannot rule out, however, the possibility that the interaction between the RNAs may be stabilized by proteins, which could have been lost or denatured during the melting experiment. Additional experiments will be required to determine what proteins are associated with U4 and U6 RNAs.

DISCUSSION

We have shown that human U4 and U6 RNAs can be recovered from dissociated snRNP particles in the form of a hybrid held together by extensive intermolecular base-pairing. The total number of distinct Sm snRNPs in mammalian cells is therefore probably only four (containing U1, U2, U5, and U4+U6 RNAs) rather than five, based on the previous assumption of one RNA species per particle (1,2). U4 RNA, like U1, U2, and U5 RNAs, must be tightly associated with one or more of the three antigenic proteins re-

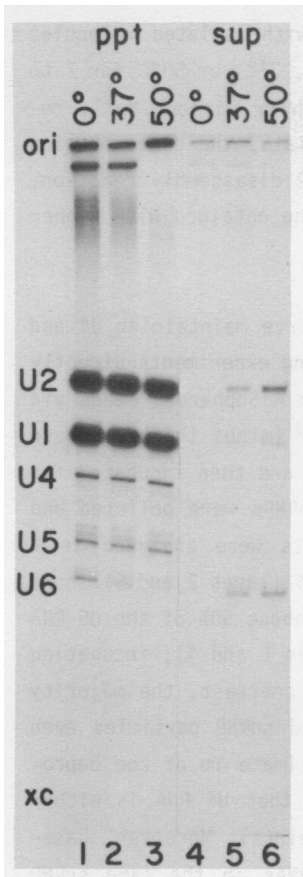

Figure 4. Release of U6 RNA from intact snRNP particles. In vivo ^{32}P-labeled snRNPs immunoprecipitated with anti-Sm antibodies bound to protein A Sepharose beads were incubated in 150 mM sodium chloride, pH 7.4, for 10 min at the temperatures (°C) indicated. After pelleting the beads, RNAs in the pellets (lanes 1-3) and supernatants (lanes 4-6) were phenol-extracted and fractionated in a denaturing 10% polyacrylamide 7M urea gel as described in Experimental Procedures.

cognized by anti-Sm antibodies (18). U6 RNA, in contrast, appears to be a constituent of Sm snRNPs only because of its ability to associate with the U4 particle.

Which of the base pairs between U4 and U6 RNAs shown in Figure 1 actually exist in snRNPs is not revealed by our data. The potential helical regions are interrupted by many loops and bulges, making calculation of a Tm value difficult (D. Crothers, personal communication). Yet, an observed Tm of about 37°C is suggestive of several stretches of contiguous nucleotides engaged in intermolecular base-pairing. Nuclease digestion studies on the complex and/or sequences of homologous RNAs from other organisms will be needed to clarify exactly which residues are in fact involved in the U4/U6 interaction.

Our identification of a U4/U6 RNA interaction in snRNPs is consistent

with a number of previous observations. 1) Both Kinlaw et al. (17,20) and we (2,16) have noticed that U4 and U6 RNAs always cofractionate during biochemical purification of snRNP complexes or when cell extracts are centrifuged in sucrose density gradients. 2) Several aspects of the U6 RNA structure had been noticed to be unusual relative to the other U RNAs found in Sm snRNPs. U6 shares neither the 5' cap structure containing $m^{2,2,7}G$ (13,14), nor a single-stranded region bounded by two hairpins near its 3' terminus (29), nor a binding site for a subset of snRNP proteins as defined by nuclease digestion studies (30). 3) In yeast, a small nuclear RNA molecule called snR 3 has been found to exhibit sequence homology to both mammalian U4 and U6 RNAs (31). Thus, snR 3 in yeast may represent an ancient evolutionary state in which the U4 and U6 sequences are covalently joined (31). The existence of separate U4 and U6 molecules could be analogous to the postulated fragmentation event which generated 5.8S and 28S rRNAs from an ancestral 23S rRNA molecule (32,33). If so, the genes for U4 and U6 RNAs may well be closely linked in higher eukaryotic species.

An intriguing question raised by our findings is how stably U6 RNA associates with U4 snRNPs inside cells. The fact that about 50% of the U6 RNA can be released from antibody-bound snRNPs by exposure to 37°C (albeit in a buffer lacking Mg^{++}) suggests that the interaction might be reversible under in vivo conditions. Low levels of uncomplexed U4 and U6 RNAs are often observed after removal of snRNP proteins, even at low temperatures (see Fig. 2A, lane 1). If we assume that snRNPs use their RNA moieties to engage in base-pairing with other nucleic acid molecules, one interesting possibility is that functionally important sequences are uncovered when the U4/U6 interaction is disrupted. In this regard it is perhaps relevant that rat U6 RNA has been reported to exist alone in structures called perichromatin granules (34), and that a small RNA required for correctly generating the 3' terminus of a sea urchin histone mRNA in Xenopus oocytes may coexist with another slightly larger small RNA in a 12S RNP (35). Knowledge of exactly which U4 and U6 residues are available for interaction with exogenous RNAs under various conditions should aid in elucidating both the role of U4 snRNPs and the contribution of U6 RNA to modulating or enhancing that activity.

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REFERENCES

1. Lerner, M.R. and Steitz, J.A. (1979) Proc. Natl. Acad. Sci. USA 76, 5495-5499.
2. Lerner, M.R., Boyle, J.A., Mount, S.M., Wolin, S.L. and Steitz, J.A. (1980) Nature 283, 220-224.
3. Yang, V.W., Lerner, M.R., Steitz, J.A. and Flint, S.J. (1981) Proc. Natl. Acad. Sci. USA 78, 1371-1375.
4. Mount, S.M., Pettersson, I., Hinterberger, M., Karmas, A. and Steitz, J.A. (1983) Cell 33, 509-518.
5. Padgett, R.A., Mount, S.M., Steitz, J.A. and Sharp, P.A. (1983) Cell 35, 101-107.
6. Rogers, J. and Wall, R. (1980) Proc. Natl. Acad. Sci. USA 77, 4471-4474.
7. Busch, H., Reddy, R., Rothblum, L. and Choi, Y.C. (1982) Ann. Rev. Biochem. 51, 617-654.
8. Branlant, C., Krol, A., Ebel, J-P., Lazar, E., Gallinaro, H., Jacob, M., Sri-Widada, J. and Jeanteur, P. (1980) Nucleic Acids Res. 8, 4143-4154.
9. Mount, S.M. and Steitz, J.A. (1981) Nucleic Acids Res. 9, 6351-6368.
10. Reddy, R., Henning, D., Epstein, P. and Busch, H. (1981) Nucleic Acids Res. 9, 5645-5658.
11. Krol, A., Branlant, C., Lazar, E., Gallinaro, H. and Jacob, M. (1981) Nucleic Acids Res. 9, 2699-2716.
12. Krol, A., Gallinaro, H., Lazar, E., Jacob, M. and Branlant, C. (1981) Nucleic Acids Res. 9, 769-787.
13. Harada, F., Kato, N. and Nishimura, S. (1980) Biochem. Biophys. Res. Commun. 95, 1332-1340.
14. Epstein, P., Reddy, R., Henning, D. and Busch, H. (1980) J. Biol. Chem. 255, 8901-8906.
15. Okada, N., Sakamoto, K., Itoh, Y. and Ohshima, Y. (1982) J. Biochem. (Tokyo) 91, 1281-1291.
16. Hinterberger, M., Pettersson, I. and Steitz, J.A. (1983) J. Biol. Chem. 258, 2604-2613.
17. Kinlaw, C.S., Robberson, B.L. and Berget, S.M. (1983) J. Biol. Chem. 258, 7181-7189.
18. Pettersson, I., Hinterberger, M., Mimori, T., Gottlieb, E. and Steitz, J.A. (1984) J. Biol. Chem., submitted.
19. Mimori, T., Hinterberger, M., Pettersson, I. and Steitz, J.A. (1984) J. Biol. Chem., in press.
20. Kinlaw, C.S., Dusing-Swartz, S.K. and Berget, S.M. (1982) Mol. Cell. Biol. 2, 1159-1166.
21. Bringmann, P., Rinke, J., Appel, B., Reuter, R. and Luhrmann, R. (1983) EMBO J. 2, 1129-1135.
22. Bringmann, P., Reuter, R., Rinke, J., Appel, B., Bald, R. and Luhrmann, R. (1983) J. Biol. Chem. 258, 2745-2747.
23. Staden, R. (1979) Nucleic Acids Res. 7, 1283-1294.
24. Hendrick, J.P., Wolin, S.L., Rinke, J., Lerner, M.R. and Steitz, J.A. (1982) Mol. Cell. Biol. 1, 1138-1149.
25. Lerner, M.R., Boyle, J.A., Hardin, J.A. and Steitz, J.A. (1981) Science 211, 400-402.
26. Maxam, A.M. and Gilbert, W. (1980) Methods Enzymol. 65, 499-560.
27. Matter, L., Schopfer, K., Wilhelm, J.A., Nyffenegger, T., Parisot, R.F. and DeRobertis, E.M. (1982) Arthritis Rheum. 25, 1278-1283.

28. Steitz, J.A. and Jakes, K. (1975) Proc. Natl. Acad. Sci. USA 72, 4734-4738.
29. Branlant, C., Krol, A., Ebel, J-P., Lazar, E., Haendler, B. and Jacob, M. (1982) EMBO J. 1, 1259-1265.
30. Liautard, J-P., Sri-Widada, J., Brunel, C. and Jeanteur, P. (1982) J. Mol. Biol. 162, 623-643..
31. Tollervey, D., Wise, J.A. and Guthrie, C. (1983) Cell 35, 753-762.
32. Clark, C.G. and Gerbi, S.A. (1982) J. Mol. Evol. 18, 329-336.
33. Walker, T.A. and Pace, N.R. (1983) Cell 33, 320-322.
34. Daskal, Y. (1981) in The Cell Nucleus, Busch, H. Ed., Vol VIII, pp. 117-137, Academic Press, New York.
35. Galli, G., Hofstetter, H., Stunnenberg, H.G. and Birnstiel, M.L. (1983) Cell 34, 823-828.