The C-group Heterogeneous Nuclear Ribonucleoprotein Proteins Bind to the 5’ Stem-Loop of the U2 Small Nuclear Ribonucleoprotein Particle*

Jamal Temsamani‡ and Thoru Pederson§

From the Cell Biology Group, Worcester Foundation for Biomedical Research, Shrewsbury, Massachusetts 01545

The C-group heterogeneous nuclear ribonucleoprotein (hnRNP) proteins bind to nascent pre-messenger RNA. In vitro studies have indicated that the C hnRNP proteins bind particularly strongly to the intron poly-pyrimidine tract of pre-mRNA and may be important for pre-mRNA splicing. In addition, there is evidence that the interaction of the C hnRNP proteins with pre-mRNA is facilitated by the U1 and U2 small nuclear RNPs (snRNPs). In the present study, we have uncovered another feature of the C hnRNP proteins that may provide a unifying framework for these previous observations; the C hnRNP proteins bind to the 5’ stem-loop of the U2 snRNP. This was detected by incubating human 32P-labeled U2 snRNPs in micrococcal nuclease-treated HeLa nuclear extracts, followed by UV-mediated protein-RNA cross-linking, which revealed that the C hnRNP proteins were cross-linked to 32P-nucleotides in the U2 snRNP. In similar experiments, no cross-linking of the C hnRNP proteins to 32P-labeled U1 or U4 snRNPs was observed. The observed cross-linking of C hnRNP proteins to U2 snRNP was efficiently competed by excess U2 RNA and by poly(U) but not by poly(A). No competition was observed with an RNA molecule comprising U2 nucleotides 105–189, indicating that the C hnRNP protein interactive regions of U2 RNA reside solely in the 5’ half of the molecule. Oligodeoxynucleotide-mediated RNase H cleavage experiments revealed that a 5’ region of U2 RNA including nucleotides 15–28 is essential for the observed C hnRNP protein cross-linking. C hnRNP protein cross-linking to U2 snRNP was efficiently competed by a mini-RNA corresponding to the first 29 nucleotides of U2 RNA, whereas no competition was observed with a variant of this mini-RNA in which the UUUU loop of stem-loop I was mutationally configured into a single-stranded RNA by replacing the stem with non-pairing nucleotides. Competition experiments with another mutant mini-U2 RNA in which the UUUU loop was replaced by AAAA indicated that both the UUUU loop and the stem are important for C hnRNP protein cross-linking, a finding consistent with other recent data on the RNA sequence specificity of C hnRNP protein binding.

The hnRNP1 proteins were discovered in 1965–1968 (1, 2) and were subsequently defined further by us and others (3–11). The advent of photochemical cross-linking methods (12, 13) and specific antibodies (14, 15) ushered in the modern era of the hnRNP field (11). Through these new experimental approaches it was soon learned that: 1) hnRNP proteins bind to nascent pre-mRNA at an early posttranscriptional stage (16, 17), 2) hnRNP proteins may support pre-mRNA splicing in vitro (18, 19), 3) hnRNP proteins are most tightly bound to the intron poly-pyrimidine tract of pre-mRNA (20, and 4) the interaction of hnRNP proteins with pre-mRNA involves a concerted role of U1 and U2 snRNPs (21). This latter finding that the interaction of hnRNP proteins with pre-mRNA involves a concerted role of U1 and U2 snRNPs (21) led us to investigate the possibility that these pre-mRNA splicing cofactors might directly interact with the hnRNP proteins independently of the interactions of hnRNP proteins with pre-mRNA. In the present investigation, we found that this is indeed the case; the C hnRNP proteins specifically bind to the 5’ stem-loop of U2 snRNP.

EXPERIMENTAL PROCEDURES

U2 snRNP Interaction with C hnRNP Proteins—32P-Labeled human U2 small nuclear RNA was transcribed from plasmid pU2G2pre (22). HeLa cell cytoplasmic S100 fractions and micrococcal nuclease-treated nuclear extracts were prepared as described previously (22–29). High specific activity [32P]U2 RNA was assembled into U2 snRNP by incubation in HeLa S100 extracts as we have described (23–25). Fifteen μl of S100-assembled U2 snRNP were then added to 15 μl of HeLa cell nuclear extract that had been pretreated with micrococcal nuclease (27) and incubated for 30 min at 30 °C. In other experiments, [32P]U1 or U4 human small nuclear RNAs were transcribed from cloned genes (23, 30, 31) and assembled into snRNPs as described (23–25) and incubated in micrococcal nuclease-treated nuclear extracts as described above.

UV-mediated Protein-RNA Cross-linking and Immunoselection—Reactions formulated and incubated as above were irradiated with 254 nm light at 4000 μW/cm² for 10 min at 4 °C (12). The samples were then incubated for 30 min at 37 °C with RNase A (25 μg/ml) and micrococcal nuclease (1500 units/ml), followed by incubation with the C hnRNP protein monoclonal antibody 4F4 (15). Immunoselection and polyacrylamide gel electrophoresis were as described previously (21, 32, 33). Parallel reactions were not irradiated and then subsequently processed similarly; all of the protein-RNA cross-linking data reported in this paper were dependent on 254 nm irradiation.

Competition Experiments—Fifteen μl of S100-assembled [32P]U2 snRNP was incubated with 15 μl of micrococcal nuclease-treated HeLa nuclear extract in the presence or absence of 2 μg of test RNA competitors. The competitors included poly(U), poly(C), poly(A), U1 RNA, and U2 RNA. An additional set of three “mini” U2 RNA wild-type and mutant competitors (see Fig. 4) were generated by T7 RNA polymerase transcription of synthetic DNA templates containing the T7 promoter and the desired U2 RNA sequence (34). Another competitor was an RNA molecule consisting of U2 nucleotides 105–189, which was transcribed from the human U2 RNA mutant plasmid pMRG3U2–50 described previously (28).

Oligodeoxynucleotide-targeted RNase H Cleavage Experiments—32P-Labeled U2 RNA was assembled in S100 and then incubated with cleoprotein; snRNP, small nuclear ribonucleoprotein.

* This work was supported by National of Institutes of Health Grant GM 21595-21. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Hybridon, Inc., One Innovation Drive, Worcester, MA 01605.
§ To whom correspondence should be addressed. Tel.: 508-842-8921, ext. 273; Fax: 508-842-7762; E-mail: werme@sci.wfbr.edu.

1 The abbreviations used are: hnRNP, heterogeneous nuclear ribonucleoprotein; snRNP, small nuclear ribonucleoprotein.
RESULTS

Fig. 1, lane 1, shows the pattern of cross-linked proteins observed when the [32P]U2 snRNP was UV irradiated in buffer alone. Fig. 1, lane 2, shows the cross-linking pattern observed when the [32P]U2 snRNP was incubated in micrococcal nuclease-treated nuclear extract. It can be seen that incubation in the nuclear extract results in the cross-linking of a group of proteins. This set of proteins is specifically selected by C hnRNP protein monoclonal antibody (Fig. 1, lane 4, compare with non-immune IgG control in lane 3). From the electrophoretic pattern of the proteins, it is clear that both the C2 and C1 hnRNP proteins are cross-linked to U2 snRNP (9, 10, 14–16, 21, 32, 33). As shown in Fig. 1, lanes 5 and 6, no cross-linking of C hnRNP proteins was observed when S100 pre-assembled [32P]U1 or U4 snRNP were incubated in nuclear extract.

Competition experiments were performed by incubating various RNAs in the nuclear extract concurrently with [32P]U2 snRNP. As shown in Fig. 2, lane 3, an excess of U2 RNA completely eliminated C hnRNP protein cross-linking to U2 snRNP. Poly(U) also competed C hnRNP protein cross-linking to U2 snRNP (Fig. 2, lane 5). In contrast, poly(A) did not compete for the binding of C hnRNP protein to U2 snRNP (Fig. 2, lane 4). Poly(C) and U1 RNA displayed intermediate levels of competition (Fig. 2, lanes 2 and 6).

To define the specific region of U2 snRNP at which the C hnRNP proteins interact, [32P]U2 snRNP was incubated with oligodeoxynucleotides complementary to various regions of U2 RNA to induce site-directed cleavage by endogenous RNase H.

The cleaved U2 snRNP was then incubated in micrococcal nuclease-treated HeLa nuclear extracts as in the preceding experiments. As shown in Fig. 3, lane 2, oligo-mediated RNase H cleavage of U2 RNA nucleotides 1–15 did not appreciably reduce C hnRNP protein cross-linking. In contrast, oligo-mediated RNase H cleavage of U2 RNA nucleotides 15–28 significantly reduced C hnRNP protein cross-linking (Fig. 3, lane 3). Oligo-mediated RNase H cleavage of nucleotides 28–42 of U2 RNA resulted in an intermediate reduction of C hnRNP protein cross-linking (Fig. 3, lane 4).

To be certain that the results shown in Fig. 3 reflect conditions of extensive oligo-mediated U2 RNA cleavage, aliquots of the reactions were removed, and deproteinized RNA was analyzed by denaturing polyacrylamide gel electrophoresis to assess the extent of oligo-mediated cleavage. As shown in Fig. 4, over 90% of the [32P]U2 RNA assembled into U2 snRNP was cleaved by the oligos complementary to nucleotides 1–15.
Extensive (>85%) U2 snRNP cleavage was also observed with the oligo complementary to nucleotides 28–42 (data not shown; see also Fig. 5A in Ref. 27).

The competition experiments (Fig. 2) suggested that a U-rich sequence element in U2 RNA is the site of C hnRNP protein interaction, and the RNase H cleavage results (Fig. 3) implicated nucleotides 15–28 as the major C hnRNP protein binding site. This region of U2 RNA includes a UUUU sequence (nucleotides 16–19). Given the strong competition of C hnRNP protein binding to U2 snRNP by poly(U) (Fig. 2) and the oligo-mediated RNase cleavage results implicating nucleotides 15–28 (or a subset of nucleotides therein) as the C hnRNP protein binding site, we investigated whether the UUUU loop in the 5′ stem-loop of U2 RNA is the C hnRNP protein binding site. We constructed the T7 RNA polymerase transcribable mini-U2 RNA templates shown in Fig. 5. Mini-U2 RNA-1 has the wild-type U2 RNA sequence; mini-U2 RNA-2 is a mutant in which stem-loop I cannot form; mini-U2 RNA-3 is a mutant in which the usual UUUU loop of the stem-loop is replaced by AAAA.

We also tested for the presence of C hnRNP protein binding sites in the 3′ half of U2 RNA by carrying out cross-linking experiments with a competitor RNA corresponding to nucleotides 105–189. No competition whatsoever was observed (Fig. 7), indicating that the C hnRNP protein interactive region is confined to the 5′ half of U2 RNA.

**DISCUSSION**

The C hnRNP proteins have a general affinity for RNA and a selective preference for pyrimidine-rich sequences, with U>C (11, 37–39). We have shown previously that the UV cross-linkable interaction of C hnRNP proteins with pre-mRNA depends on the integrity of U1 and U2 snRNPs (21). Our results had suggested that C hnRNP proteins might facilitate juxtaposition of U1 or U2 snRNPs on the one hand and pre-mRNA on the other. From the nucleotide sequences of U1 and U2 snRNAs, we reasoned that the latter constituted a more likely C hnRNP protein binding target because U2 snRNA has a 5′
stem-loop structure that contains a UUUU loop, whereas U1 snRNP has no U-rich sequences other than the Sm domain, which is tightly complexed with protein (40, 41). We, therefore, asked in the present investigation whether there is a binding interaction between the C hnRNP proteins and U2 snRNP (and also, in the course of these experiments, asked the same question with respect to U1 and U4 snRNPs). Our results show that the C hnRNP proteins specifically bind to the 5’ stem-loop of U2 snRNP and further demonstrate that the UUUU loop at this position in U2 RNA is the primary determinant of C hnRNP protein binding.

We do not know if stem-loop I is the sole C hnRNP protein binding site in U2 snRNP. However, the lack of C hnRNP cross-linking competition seen with the 3’ half of U2 RNA (Fig. 7) indicates that all C hnRNP protein binding is restricted to nucleotides 1–104 of U2 RNA. Thus, although it is clear that a major C hnRNP protein binding site is nucleotides 15–28 (stem-loop I of U2 snRNP), we cannot at present rule out additional C hnRNP protein binding sites lying between nucleotides 29 and 104.

It is of interest that the mini-U2 RNA in which the UUUU loop was replaced by AAAA, but with retention of the wild-type stem of stem-loop I, showed partial competition, indicating that the stem itself may contribute to the binding of C hnRNP protein. This is of considerable interest since this stem has been implicated in pre-mRNA splicing (42). The very efficient competition of C hnRNP binding to U2 snRNP we observed with poly(U), which contains extensive secondary structure (43) but is obviously devoid of base paired stems, probably reflects a target attribute of the poly(U) competitor. The RNA-binding elements of the C1 and C2 hnRNP proteins only occupy 7 ± 1 nucleotides (44). Thus, poly(U) probably represents an enormous array of targets per molecule which, on the basis of available binding data (44, 45), would be expected to titrate C hnRNP proteins very efficiently. This suggests that the binding affinity of the 5’ stem-loop of U2 RNA for C hnRNP proteins arises from the UUUU loop acting in conjunction with the stem and that the use of poly(U) as a competitor is essentially an unnatural situation (see also Ref. 45). Since the C hnRNP protein contact site in RNA is 7 ± 1 nucleotides and since in vitro selection of optimal RNA ligands for C1 hnRNP protein binding generates “winners” containing U stretches of five or more, with maximal affinity seen at (U)14 (46), it is perhaps not surprising that when the contiguous U stretch is only four, as in stem-loop I of U2 RNA, there is a need for additional binding energy conferred by vicinal sequences, i.e. the stem.

A U2 snRNP-specific human autoantibody immunoprecipitates a set of proteins (47) later shown to be stoichiometric components of the biochemically purified U2 snRNP particle (48, 49). These do not include the C hnRNP proteins, suggesting that in the steady-state only a small fraction of U2 snRNP is associated with C hnRNP proteins. It should be borne in mind that these previous protein composition studies of U2 snRNP were based on the major 11–17 S fraction of nuclear U2 snRNP that does not fractionate with endogenous spliceosomes. Thus, our results are compatible with a short-lived, dynamic, UV cross-linkable association of C hnRNP proteins with U2 snRNP in the spliceosome, perhaps linked to the cyclical assembly-disassembly of the splicing apparatus on each pre-mRNA.

In previous cytological-immunocytochemical work, we temporally and spatially linked the U2 snRNP particle to chromosomal sites of intense pre-mRNA transcription and splicing (50). A subsequent study indicated that the C hnRNP proteins are initially assembled onto nascent transcripts independently of snRNPs (51), confirming our earlier results on the posttranscriptional immediacy with which hnRNP proteins bind to pre-mRNA (52, 53). These previous results, together with the present investigation, suggest that C hnRNP proteins first bind to pre-mRNA and subsequently interact in binary complexes with U2 snRNP arriving in the forming spliceosome.

Despite considerable effort by several groups, the function of the C hnRNP proteins in gene expression still remains unclear. We have recently connected the pre-mRNA binding activity of the C1 hnRNP protein to its phosphorylation (32) and have further implicated U6 small nuclear RNA in this covalent modification (33). The base pairing interaction of U2 and U6 snRNPs (42, 54, 55) and our recent connection of C hnRNP protein phosphorylation and U6 RNA (33) add further weight to the concept of a C hnRNP protein interaction between U2 snRNP and pre-mRNA, possibly also involving U6 RNA as a modulator of C hnRNP protein phosphorylation. The present investigation, together with our previous results, suggests that C hnRNP proteins bind both to U2 snRNP and pre-mRNA and may thus facilitate the spatial juxtaposition of pre-mRNA into a configuration favorable for splicing via hnRNP protein-protein interactions. Although there is ample evidence for snRNP:snRNP interactions in the spliceosome (56), the energetically demanding folding of pre-mRNA needed to align the 5’ and 3’ splice sites of each intron over long distances may require facilitation by additional, non-snRNP components. Our results suggest that the C hnRNP proteins may contribute to this requirement in the formative spliceosome. In this respect, it is noteworthy that a binding site for another hnRNP protein, A1, has recently been identified in the U5 snRNP (57), indicating that hnRNP-snRNP interactions may be a general feature of the assembling splicing apparatus.

Acknowledgments—We thank Molly Rhoadhouse for skilful assistance, and we are particularly grateful for the advice of Sandra Mayrand. 4F4 antibody was provided by Serafin Pfitzler-Roma and Gideon Dreyfuss, Howard Hughes Medical Institute, University of Pennsylvania School of Medicine.

REFERENCES

1. Samarina, O. P., Arstian, I. S., and Georgiev, G. P. (1965) Doklady Akad. Nauk USSR 163, 1510–1513
2. Samarina, O. P., Lukanidin, E. M., Molnar, J., and Georgiev, G. P. (1968) J. Mol. Biol. 33, 251–263
3. Bhorjee, J. S., and Pederson, T. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 3345–3349
4. Pederson, T. (1974) J. Mol. Biol. 83, 163–183
5. Pederson, T. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 617–621
6. Quinlan, T., Billings, P. B., and Martin, T. E. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 2632–2636
7. Kato, V. M., and Pederson, T. (1975) J. Mol. Biol. 95, 227–238
8. Firtel, R. A., and Pederson, T. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 301–305
9. Bryer, A. L., Christensen, M. E., Walker, B. W., and LeStourgeon, W. M. (1977) Cell 11, 127–138
10. Karn, J., Vidoli, G., Boifa, L. C., and Alifrey, V. G. (1977) J. Biol. Chem. 252, 7307–7322
11. Pederson, T. (1983) J. Cell Biol. 97, 1321–1326
12. Mayrand, S. H., and Pederson, T. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 2209–2212
13. Mayrand, S. H., Setyono, B., Greenberg, J. R., and Pederson, T. (1981) J. Cell Biol. 90, 380–384
14. Leeser, G. P., Erecar-Willek, J., and Martin, T. E. (1984) J. Biol. Chem. 259, 1827–1833
15. Choi, Y. D., and Dreyfuss, G. (1984) J. Cell Biol. 99, 1997–2004
16. Economides, I. V., and Pederson, T. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 1599–1602
17. Faks, S., Leeser, G., and Martin, T. E. (1986) J. Cell Biol. 103, 1153–1157
18. Choi, Y. D., Grabowski, P. J., Sharp, P. A., and Dreyfuss, G. (1986) Science 231, 1534–1539
19. Szczeklikowska, H., Szer, W., Furdon, P. J., and Kole, R. (1986) Nucleic Acids Res. 14, 5241–5254
20. Swanson, M. S., and Dreyfuss, G. (1988) RIBMO J. 7, 3519–3529
21. Mayrand, S. H., and Pederson, T. (1980) Nucleic Acids Res. 8, 3307–3318
22. Kleinschmidt, A. M., and Pederson, T. (1987) Mol. Cell. Biol. 7, 3131–3137
23. Patton, J. R., Patterson, R. J., and Pederson, T. (1987) Mol. Cell. Biol. 7, 4050–4057
24. Patton, J. R., and Pederson, T. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 747–751
25. Kleinschmidt, A. M., Patton, J. R., and Pederson, T. (1989) Nucleic Acids Res. 17, 4817–4828
26. Patton, J. R., Habets, W., van Venrooij, W. J., and Pederson, T. (1989) Mol. Cell. Biol. 9, 3360–3368
27. Temsamani, J., Rhoadhouse, M., and Pederson, T. (1991) J. Biol. Chem. 266, 20356–20362
28. Jacobson, M. R., Rhoadhouse, M., and Pederson, T. (1993) Mol. Cell. Biol. 13, 1119–1129
29. Patton, J. R., Jacobson, M. R., and Pederson, T. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 3324–3328
30. Hammerstrom, K., Westin, G., and Pettersson, U. (1982) EMBO J. 1, 737–739
31. Madore, S. J., Wieben, E. D., Kunkel, G. R., and Pederson, T. (1984) J. Cell Biol. 99, 1140–1144
32. Mayrand, S. H., Dwen, P., and Pederson, T. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7764–7768
33. Mayrand, S. H., Fung, P., and Pederson, T. (1996) Mol. Cell. Biol. 16, 1241–1246
34. Milligan, F., Groebe, D. R., Witherell, G. W., and Uhlenbeck, O. C. (1987) Nucleic Acids Res. 15, 8783–8789
35. Agrawal, S., Mayrand, S. H., Zamecnik, P. C., and Pederson, T. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1401–1405
36. Temsamani, J., Agrawal, S., and Pederson, T. (1991) J. Biol. Chem. 266, 468–473
37. Pullman, J. M., and Martin, T. E. (1983) J. Cell Biol. 97, 99–111
38. Swanson, M. S., and Dreyfuss, G. (1988) Mol. Cell. Biol. 8, 2237–2241
39. Gorlach, M., Witzekind, M., Beckman, R. A., Mueller, L., and Dreyfuss, G. (1992) EMBO J. 11, 3289–3295
40. Reddy, R., and Busch, H. (1988) in Small Nuclear Ribonucleoprotein Particles (M. L. Birnstiel, ed.), pp. 1–37, Springer-Verlag, Berlin
41. Guthrie, C., and Patterson, B. (1988) Ann. Rev. Genet. 22, 387–419
42. Wu, J., and Manley, J. L. (1992) Mol. Cell. Biol. 12, 5464–5473
43. Davies, D. R., and Felsenfeld, G. (1968) in: Structural Chemistry and Molecular Biology (A. Rich and N. Davidson, eds), pp. 422–429, W. H. Freeman and Co., San Francisco
44. Amrute, S. B., Abdul-Manan, Z., Pandey, V., Williams, K. R., and Modak, M. J. (1994) Biochemistry 33, 8282–8291
45. McAfee, J. G., Soltaninassah, S. R., Lindsay, M. E., and LeStourgeon, W. M. (1996) Biochemistry 35, 1212–1222
46. Gorlach, M., Burd, C. G., and Dreyfuss, G. (1994) J. Biol. Chem. 269, 23074–23078
47. Mimori, T., Hinterberger, M., Pettersson, I., and Steitz, J. A. (1984) J. Biol. Chem. 259, 560–565
48. Lührmann, R., Kastner, B., and Bach, M. (1990) Biochim. Biophys. Acta 1087, 265–292
49. Behrens, S. E, Tye, K., Kastner, B., Reichelt, R., and Lührmann, R. (1993) Mol. Cell. Biol. 13, 307–319
50. Sass, H., and Pederson, T. (1984) J. Mol. Biol. 180, 911–926
51. Amore, S. A., Raychaudhuri, G., Cass, C. L., van Venrooij, W. J., Habets, W. J., Krainer, A. R., and Beyer, A. L. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8409–8413
52. Economides, I. V., and Pederson, T. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 1469–1472
53. Economides, I. V., and Pederson, T. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 1599–1602
54. Wu, J., and Manley, J. L. (1991) Nature 352, 818–821
55. Datta, B., and Weiner, A. M. (1991) Nature 352, 821–824
56. Moore, M. J., Query, C. C., and Sharp, P. A. (1993) in The RNA World (R. F. Gesteland and J. F. Atkins, eds), pp. 303–357, Cold Spring Harbor Laboratory, Plainview, NY
57. Black, A. C., Luo, J., Watanabe, C., Chun, S., Bakker, A., Fraser, J. K., Morgan, J. P., and Rosenblatt, J. D. (1995) J. Virol. 69, 6852–6858