The EMBO Journal vol.10 no.9 pp.2645-2651, 1991

An intact Box C sequence in the U3 snRNA is required for binding of fibrillarin, the protein common to the major family of nucleolar snRNPs

Susan J.Baserga, Xiangdong W.Yang and Joan A.Steitz

Department of Molecular Biophysics and Biochemistry, Howard Hughes Medical Institute, Yale School of Medicine, 333 Cedar St, New Haven, CT 06511, USA

Communicated by J.A.Steitz

The mammalian U3 snRNP is one member of a recently described family of nucleolar snRNPs which also includes U8, U13, U14, X and Y. All of these snRNPs are immunoprecipitable by anti-fibrillarin autoantibodies, suggesting the existence of a common binding site for the 34 kDa fibrillarin (Fb) protein. Two short nucleotide sequences, called Boxes C and D, present in each of these RNAs are the most likely sites for fibrillarin binding. We have developed a HeLa *in vitro* assembly system for binding of fibrillarin to human U3 snRNA. Reconstitution of the input RNA is specific in our assay since four of the other nucleolar small RNAs (U8, U13, X and Y) which have Boxes C and D become immunoprecipitable by anti-fibrillarin whereas two RNAs which lack these sequences (5S and 5.8S) do not. Deletion analyses of the U3 snRNA demonstrate that the presence of Box C but not Box D is required for fibrillarin binding. Moreover, seven single or double site-specific mutations in the U3 Box C abolish binding. The role of the Box C-fibrillarin interaction in the biogenesis of the Fb snRNPs is discussed.

Key words: fibrillarin/nucleolus/snRNPs/U3 snRNA

Introduction

U1, U2, U3, U4, U5 and U6 constitute the major small nuclear ribonucleoprotein particles (snRNPs) in mammalian cells because of their high abundance ($0.2-1\times10^6$ copies/cell). Each snRNP consists of one highly structured RNA and as many as 10 different proteins. With the exception of U3, all of these snRNPs participate in pre-mRNA splicing and can be found in spliceosomes in the nucleoplasm. The spliceosomal snRNPs, except for U6, are directly immunoprecipitable by anti-Sm sera, which recognize a common epitope on several of the particle proteins (reviewed in Luhrmann, 1988; Zieve and Sauterer, 1990). In contrast, the U3 snRNP participates in rRNA processing (Kass *et al.*, 1990; Savino and Gerbi, 1990) and is located in the nucleolus (Nakamura *et al.*, 1968; Weinberg and Penman, 1968; Prestayko *et al.*, 1970; Tyc and Steitz, 1989). It is immunoprecipitable by antibodies directed against an abundant 34 kDa nucleolar protein (Lischwe *et al.*, 1985) called fibrillarin, and not by anti-Sm sera. The U3 snRNP therefore belongs to a distinctly different class than the spliceosomal snRNPs by virtue of its function, subcellular location, and protein composition.

The biogenesis of the snRNPs of the Sm class has been well-studied. The snRNAs, except for U6, possess a short conserved sequence [PuA(U)$_n$GPu] which was originally shown to be the binding site for the Sm proteins (B', B, D, E, F, G) by RNase digestion of native snRNP particles (Liautard *et al.*, 1982). Specific mutations studied both *in vivo* and *in vitro* confirmed the importance of this sequence for binding of the Sm proteins (Hernandez and Weiner, 1986; Mattaj, 1986; Hamm *et al.*, 1987; Surowy *et al.*, 1989). In addition to the common Sm proteins, each snRNP binds specific proteins. For example, the U1 snRNP contains three specific proteins called the 70K, A and C. The arrangement of these proteins in the U1 RNP has been determined using: (i) *in vitro* assembly of *in vitro* synthesized U1 RNA with HeLa cell extracts (Patton *et al.*, 1987; Patton and Pederson, 1988; Lutz-Reyermuth and Keene, 1989; Scherly *et al.*, 1989; Surowy *et al.*, 1989); (ii) *in vitro* assembly of *in vitro* synthesized U1 RNA with *Xenopus* oocyte extracts (Hamm *et al.*, 1987, 1988; Scherly *et al.*, 1989); (iii) direct binding of U1 RNA to U1-specific proteins synthesized *in vitro* (Spritz *et al.*, 1987; Query *et al.*, 1989; Scherly *et al.*, 1989; and Surowy *et al.*, 1989); and (iv) expression of mutated U1 genes injected into *Xenopus* oocytes (Hamm *et al.*, 1990b). Further studies of the assembly of the U1 snRNP in *Xenopus* oocytes and in mammalian cells have localized the steps in its biogenesis to various cell compartments (Feeney *et al*, 1989; Fischer and Lührmann, 1990; Hamm *et al.*, 1990a; Neuman de Vegvar and Dahlberg, 1990). After transcription by RNA polymerase II in the nucleus, U1 RNA quickly exits to the cytoplasm where it binds pre-assembled Sm proteins and its 7-methyl guanosine cap undergoes modification to become trimethylated (2,2,7-trimethyl guanosine). Only then is the nascent U1 particle imported into the nucleus and its assembly completed by binding of the U1-specific proteins. Import and trimethylation are not affected by deletion of the binding sites for the specific proteins from U1 RNA, whereas the Sm binding site is crucial for these processes.

In contrast to the U1 snRNP, very little is known about the assembly of the U3 snRNP. The U3 snRNP consists of one 217 nucleotide-long trimethyl guanosine-capped RNA and six proteins (74, 59, 34, 30, 13 and 12.5 kDa; Parker and Steitz, 1987). The 34 kDa protein, fibrillarin, is common to at least five other nucleolar snRNPs (U8, U13, U14, X and Y; Tyc and Steitz, 1989; Liu and Maxwell, 1990). Except for fibrillarin, the protein components of these other nucleolar snRNPs are not yet known. The U3, U8, U13 and U14 RNAs possess two short nucleotide sequences called Boxes C and D (see Figure 1a) which are also conserved throughout U3 evolution (Wise and Weiner, 1980; Hughes *et al.*, 1987; Jeppesen *et al.*, 1988; Tyc and Steitz, 1989). RNAs X and Y also contain Box D and the most highly conserved first six nucleotides of the Box C sequence (UGAUGA and UGAAGA, respectively; K.Tyc, personal communication). Immunoprecipitation with anti-fibrillarin

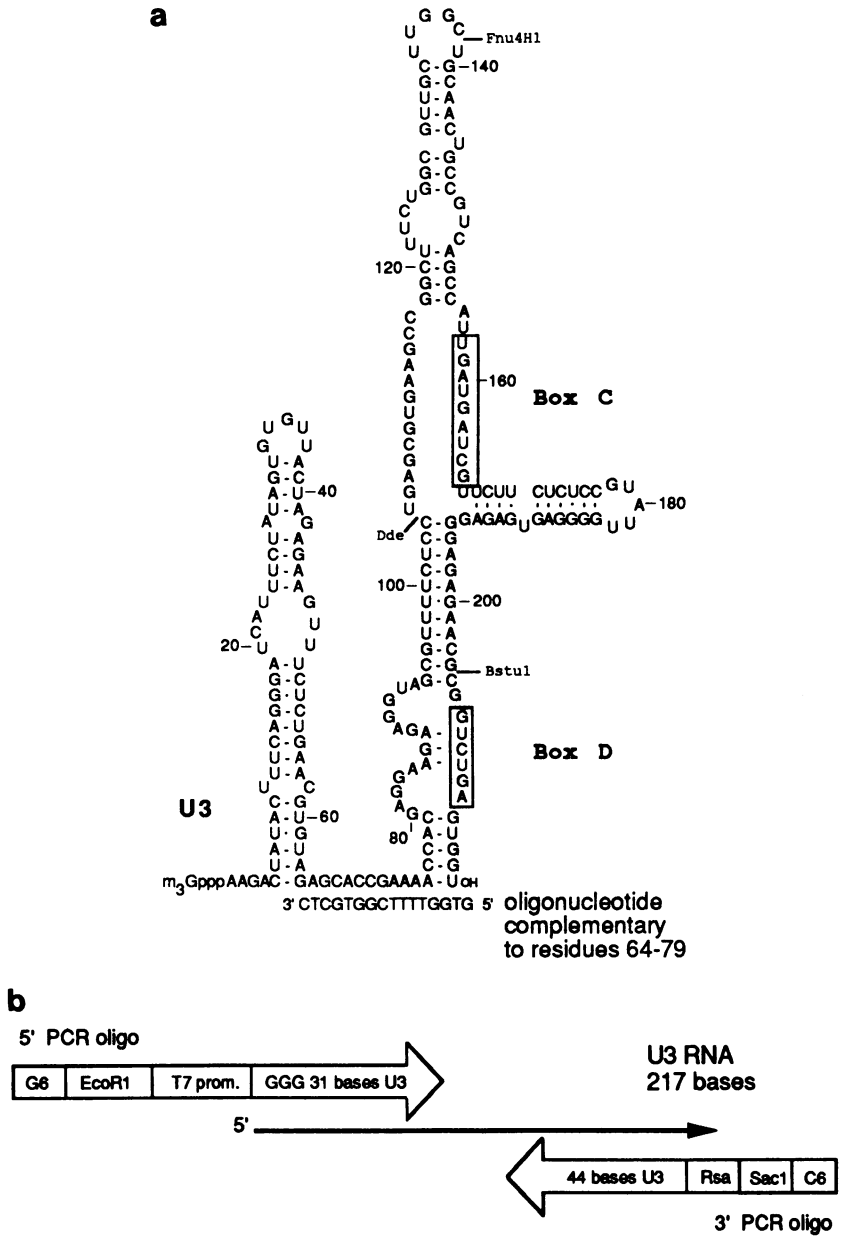

Fig. 1. a. Primary and secondary structure of human U3 RNA. The U3 structure is based on analyses using chemical and enzymatic probes (Parker and Steitz, 1987). The conserved Box C and Box D regions (Jeppesen *et al.*, 1988) are indicated. A U3 fragment containing nucleotides 80–217 was generated by RNase-H directed digestion of native U3 using the oligonucleotide shown (see text). **b**. Cloning of the human U3 snRNA for *in vitro* synthesis. A human U3 RNA was cloned under the control of a T7 RNA polymerase promoter by PCR. A schematic of the 5′ and 3′ oligonucleotides is shown. The 5′ oligonucleotide has at its 3′ end 31 nucleotides of U3 RNA sequence, preceded by three guanosines to facilitate the start of T7 transcription, the T7 RNA polymerase promoter, an *Eco*RI site for cloning, and 6 guanosines to serve as a 'clamp' for *Eco*RI cleavage. The 3′ oligonucleotide has at its 3′ end 44 bases complementary to the U3 RNA preceded by an *Rsa*I site (to yield the exact 3′ end of the U3 RNA), a *Sac*I site for cloning and six cytidines to serve as a clamp for cleavage by *Sac*I.

antibodies following RNase digestion of the U3 snRNP has suggested that Boxes C and D interact (directly or indirectly) with the fibrillarin protein (Parker and Steitz, 1987).

As the first step toward a better understanding of the assembly of the U3 snRNP, we have undertaken a mutational analysis of conserved Boxes C and D to establish their role in fibrillarin binding. Because U3 and five other nucleolar snRNAs share sequence similarity, specific protein binding properties and subcellular location, our results are relevant to this entire class of snRNPs, which we propose be called the Fb snRNPs.

Results

Binding of U3 RNA to fibrillarin in HeLa cell extracts

We first tested the ability of fibrillarin to assemble with U3 RNA *in vitro* using native U3 isolated from HeLa cells and labeled with ^{32}pCp. After incubation for 45 min at 37°C in a HeLa whole cell extract (WCE), fibrillarin binding was determined by immunoprecipitation with anti-fibrillarin antibodies; since the antibody does not bind the RNA alone (data not shown; Parker and Steitz, 1987), the amount of U3 RNA immunoprecipitated is an indication of assembly.

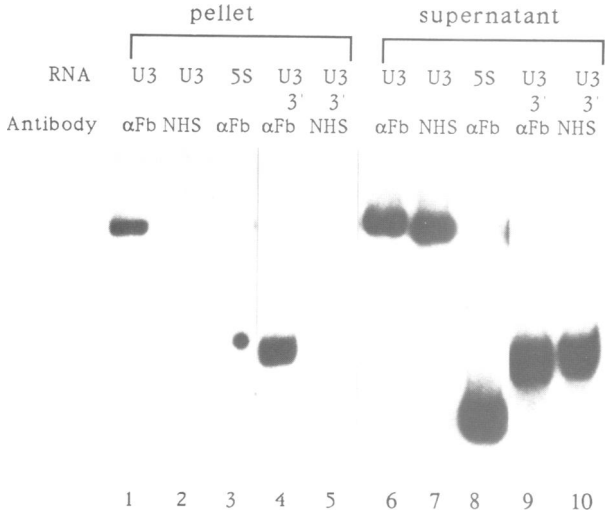

Fig. 2. Specific binding of fibrillarin to U3 RNA in HeLa whole cell extracts (WCE). ^{32}pCp-labeled RNAs from HeLa cells were incubated in HeLa WCE for 45 min at 37°C and then analyzed for fibrillarin binding by immunoprecipitation with either an anti-fibrillarin serum (αFb) or normal human serum (NHS) as a control. The pellets (lanes 1–5) and corresponding supernatants (lanes 6–10) were analyzed on an 8% denaturing polyacrylamide gel. Lanes 1 and 6: U3 RNA precipitated with anti-fibrillarin antibodies. Lanes 2 and 7: U3 RNA precipitated with NHS. Lanes 3 and 8: 5S RNA precipitated with anti-fibrillarin antibodies. Lanes 4 and 9: U3 RNA 3′ fragment nt 80–217 (see text) precipitated with anti-fibrillarin antibodies. Lanes 5 and 10: U3 RNA fragment nt 80–217 (see text) precipitated with NHS.

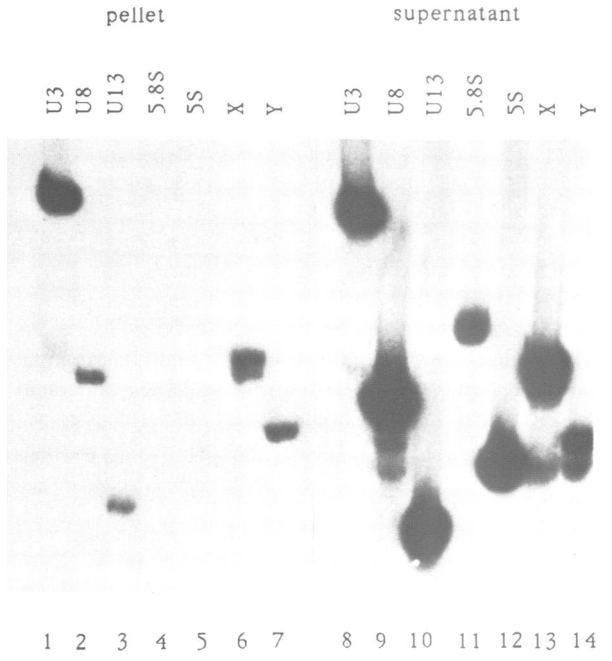

Fig. 3. Five Fb snRNAs bind fibrillarin *in vitro*. ^{32}pCp RNAs from HeLa cells were incubated in HeLa WCE for 45 min at 37°C and then analyzed for fibrillarin binding by immunoprecipitation with anti-fibrillarin serum. The pellets (lanes 1–7) and corresponding supernatants (lanes 8–14) were analyzed on an 8% denaturing polyacrylamide gel. Lanes 1 and 8: U3 RNA. Lanes 2 and 9: U8 RNA. Lanes 3 and 10: U13 RNA. Lanes 4 and 11: 5.8S rRNA. Lanes 5 and 12: 5S rRNA. Lanes 6 and 13: RNA X. Lanes 7 and 13: RNA Y.

As shown in Figure 2, lane 1, fibrillarin binds to U3 RNA added to the HeLa WCE. The interaction is specific since normal human serum (NHS) does not immunoprecipitate U3 (Figure 2, lane 2) and ^{32}pCp-labeled 5S RNA isolated from HeLa cells is not immunoprecipited by anti-fibrillarin antibodies (Figure 2, lane 3). The percentage reconstitution (pellet/pellet + supernatant) of U3 RNA varied from 10 to 50% in different experiments.

To determine which part of U3 participates in fibrillarin binding, a shortened U3 RNA was created by oligonucleotide-directed RNase H digestion of ^{32}pCp-labeled full-length U3 isolated from HeLa cells. The oligonucleotide is complementary to bases 64–79 of the U3 RNA (see Figure 1a); upon digestion with RNase H an RNA containing the 3′ 138 nucleotides of U3 is created. This U3 fragment, containing conserved Boxes C and D (see Figure 1a), binds fibrillarin to approximately the same extent as the intact full-length U3 (Figure 2, lane 4). This suggests that the sequences required for fibrillarin binding are contained in the 3′ 138 nucleotides of U3.

Several other types of cell extracts were tested for fibrillarin binding to U3 RNA. The HeLa WCE was found to be several-fold more active than HeLa S100 or HeLa Dignam extract (Dignam *et al.*, 1983; Heintz and Roeder, 1984). Fibrillarin binding in HeLa WCE was comparable to that observed in mouse ascites S100 (data not shown), an extract known to be active in the first cleavage step of rRNA processing (Kass *et al.*, 1990). Reconstitution with fibrillarin does not require exogenously added ATP or creatine phosphate (data not shown). The WCE was most active when the protein concentration exceeded 30 mg/ml (data not shown).

Other Fb snRNAs also bind fibrillarin in vitro

The results shown in Figure 2 demonstrate that U3 binds fibrillarin in WCE, that this interaction is specific, and that the 3′ 138 residues of U3, which contain Boxes C and D, are sufficient. Since there are at least five other snRNAs in mammalian cells which are both immunoprecipitable by anti-fibrillarin sera and contain Box C and D homologs (Tyc and Steitz, 1990; K.Tyc, personal communication), we tested their ability to bind fibrillarin *in vitro*. Four of these (U8, U13, X, and Y) and two RNAs which do not bind fibrillarin (5S, 5.8S) were isolated from HeLa cells, labeled with ^{32}pCp and incubated in HeLa WCE. The immunoprecipitation results are shown in Figure 3. All of the RNAs which belong to the Fb snRNP class (U3, U8, U13, X, Y) become bound by fibrillarin in WCE (lanes 1–3, 6–7) while neither 5.8S rRNA nor 5S rRNA does (lanes 4 and 5).

Are conserved Boxes C and D in U3 RNA necessary for fibrillarin binding?

In order to transcribe and assay mutant U3 RNA molecules for their interaction with the fibrillarin protein, we cloned a human U3 cDNA under the control of a T7 RNA polymerase promoter. This was accomplished starting with purified HeLa U3 RNA using a procedure involving PCR which has general cloning applications (see Figure 1b). The U3 cDNA clone was designed so that run-off transcription of *Rsa*I digested DNA would yield an RNA with a 3′ end identical to that of the wild-type U3 and a 5′ end with only three additional guanosine residues. Furthermore, after subcloning into M13mp18 and subsequent mutagenesis, the

replicative form M13 DNA can be used directly as a template for T7 transcription without subcloning back into the parent pSP64 plasmid.

To assess the roles of Boxes C and D in fibrillarin binding, we first tested deletions of the 3′ end of the U3 RNA, as shown in Figure 4a. We compared RNAs transcribed from the U3 cDNA clone cut with *Rsa*I (full-length), *Bst*UI (ΔD), *Fnu*4HI (ΔCD1) or *Dde*I (ΔCD2) (see Figure 1a). Upon immunoprecipitation after incubation in WCE (Figure 4b), only the full-length U3 and the U3 ΔD were observed to bind fibrillarin (lanes 1–2). The two RNAs with Box C and Box D deleted (ΔCD1 and ΔCD2) did not (lanes 3–4). These results suggest that Box D is not involved in fibrillarin binding, whereas Box C remains a candidate.

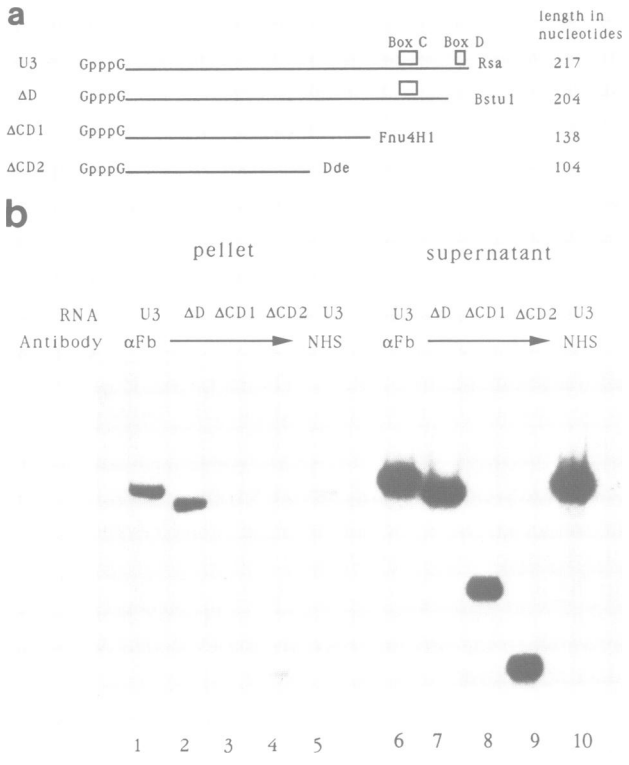

Fig. 4. a. Conserved region Box C is necessary for fibrillarin binding *in vitro*. Truncations of the U3 RNA were generated by digestion of the template DNA with the indicated restriction enzymes. Digestion by *Rsa*I yields a U3 RNA with the correct 3′ end after T7 transcription. Digestion with *Bst*UI yields an RNA missing Box D (ΔD) which is 204 nucleotides in length. Digestion with *Fnu*4HI yields an RNA 138 nucleotides long that is missing Boxes C and D (ΔCD1). Digestion with *Dde*I yields an RNA 104 nucleotides long missing Boxes C and D (ΔCD2). There are three extra guanosines at the 5′ end of each of these transcripts. **b.** The ^{32}pCp RNAs represented in (a) were incubated in HeLa WCE for 45 min at 37°C and analyzed for binding to fibrillarin by immunoprecipitation with anti-fibrillarin antibodies (αFb or NHS). The pellets (lanes 1–5) and corresponding supernatants (6–10) from the immunoprecipitations were run on an 8% denaturing polyacrylamide gel. The U3 RNA (lane 1) and the U3 RNA lacking Box D (ΔD; lane 2) bind to fibrillarin whereas two RNAs which lack both Boxes C and D do not (ΔCD1, lane 3, and ΔCD2, lane 4).

The possibility that fibrillarin binding is directed by Box C was further tested by analyzing the effect of specific base mutations in Box C. Seven U3 RNAs with either single or double base substitutions (outlined in Table I) were incubated in WCE and immunoprecipitated with anti-fibrillarin antibodies. Figure 5 shows that while wild-type U3 RNA binds fibrillarin (lane 1), all seven RNAs with Box C mutations bind fibrillarin to a much lesser extent (lanes 2–8) (ranging from 5 to 10% of wild-type binding). This level is about the same as seen for the background binding of fibrillarin to U3 RNA upon immunoprecipitation with NHS (lane 9). We conclude that an intact Box C is required for fibrillarin binding. In contrast, four single base substitutions or a single base deletion in Box D and a substitution of the loop at U3 residues 134–138 (see Figure 1 and Materials and methods) have no effect on fibrillarin binding (data not shown).

Discussion

We have developed an *in vitro* assembly system for binding of human U3 RNA to fibrillarin using HeLa WCE. Reconstitution of the input RNA with fibrillarin is specific in our assay since four other Fb snRNAs (U8, U13, X, Y) which have Boxes C and D become immunoprecipitable by anti-fibrillarin serum whereas two RNAs which lack these sequences (5S and 5.8S) do not. Deletion analyses using fragments of *in vivo* or *in vitro* transcribed U3 RNA demonstrate that the presence of Box C but not Box D is required for fibrillarin binding. Moreover, seven single or double site-specific mutations in Box C abolish binding. We conclude that an intact Box C is necessary for fibrillarin binding to U3 snRNA.

Since the Box C sequence is conserved among all known mammalian Fb snRNPs (Tyc and Steitz, 1989; Liu and Maxwell, 1990), it seems likely that it plays a similar role in directing the assembly of the fibrillarin protein into each of these nucleolar snRNPs. For U3, U8 and U13, computer modelling suggests that the Box C sequence occurs in a single-stranded region bounded on its 3′ side by a stem–loop. Thus, these Fb snRNPs share both sequence identity for Box C and surrounding structural similarity. The precise length of the Box C sequence in RNAs X and Y remains to be defined; these RNAs do contain six nucleotides

Table I. Site-directed mutagenesis of Box C in U3 snRNA

Mutation	Oligonucleotide used for mutagenesis	Box C sequence
wt.		UGAUGAUCG
U158C	5′-GAAGAACGATCATC**G**ATGGCTG	C**G**AUGAUCG
G159A	5′-GAAGAACGATCATT**A**ATGGCTG	U**A**AUGAUCG
A160C	5′-GAAGAACGATCA**G**CAATGGCTG	UG**C**UGAUCG
GA159UC	5′-GAAGAACGATCA**GA**AATGGCTG	U**UC**UGAUCG
G162A	5′-GAAGAACGATT**A**TCAATGGCTG	UGAU**A**AUCG
A163U	5′-GAAGAACGAA**A**CATCAATGGCTG	UGAUG**U**UCG
CG165AU	5′-GAAGAA**TA**ATCATCAATGGCTG	UGAUGAU**AU**

of the Box C sequence (UGAUGA and UGAAGA, respectively; K.Tyc, personal communication). Further evidence that Box D is not necessary for fibrillarin binding comes from studies of a trypanosome RNA, RNAB, of unknown function (Hartshorne and Agabian, 1991). This snRNA bears a putative six nucleotide Box C sequence (UGAUGA) but no Box D sequence and is immunoprecipitable by anti-fibrillarin sera.

In accord with the conservation of Box C among Fb snRNAs, the fibrillarin protein is highly conserved among species. The yeast (Schimmang *et al.*, 1989; Henriquez *et al.*, 1990), *Xenopus* (Lapeyre *et al.*, 1990) and human (Jansen *et al.*, 1991) fibrillarin genes have been cloned. Human and yeast fibrillarin are 70% identical and 80% similar while human and *Xenopus* fibrillarin are 81% identical. Furthermore, human fibrillarin can functionally complement a yeast fibrillarin mutant (Jansen *et al.*, 1991). This conservation argues for an important function for fibrillarin.

We have shown that Box C is necessary for the binding of fibrillarin to U3 snRNA. Is the presence of Box C alone sufficient? We suspect that it may not be and that some aspect of the 3′ stem−loop structure of U3 snRNA may also be critical. In Figure 2 we observed that shortened U3 RNAs containing only the 3′ 138 residues can bind to fibrillarin in our *in vitro* assay. However, a 3′ end fragment created by RNase H digestion of native U3 snRNA that is only 97 residues long (U3 nt 121−217) does not assemble (data not shown), even though it contains the Box C sequence. This suggests that the region between nucleotides 80 and 121 of U3 (see Figure 1a) is required to confer a particular conformation on the Box C sequence which may be necessary for fibrillarin binding. An alternative possibility is that fibrillarin interacts directly with other parts of the U3 snRNA and that these sequences stabilize its binding. However, such sequences might be expected to also appear in the other Fb snRNAs, whose conservation is limited to Boxes C and D.

Does fibrillarin bind directly to Box C? In our assay we cannot distinguish between direct binding of fibrillarin to Box C and indirect binding through another Fb snRNP protein. There are at least five other proteins in the U3 snRNP, some of which may be common to the other Fb snRNPs. The observation that U3 immunoprecipitability by anti-fibrillarin antibodies is reduced in 0.5 M NaCl (Parker and Steitz, 1987; Tyc and Steitz, 1989) suggests that the interaction may indeed be indirect, but U8 and U13 remain immunoprecipitable at the same salt concentration, arguing against this explanation. Experiments testing association of the U3 RNA with fibrillarin in the absence of other proteins will be necessary to resolve this question.

Most studies analyzing specific snRNA−protein interactions have tested large deletions or substitutions in the RNA instead of point mutations (for reviews, see Luhrmann, 1988; Zieve and Sauterer, 1990). In the studies where point mutations in the RNA have been analyzed, different proteins have exhibited quite different sensitivities to mutation in their binding sites. For example, Scherly *et al.* (1990) studied the binding of the U1A and U2B″ proteins to their respective RNAs, U1 and U2, and found that exchange of only two nucleotides between the two RNAs reverses the binding specificity of these proteins. Yuo and Weiner (1989) found that two single nucleotide substitutions in stem−loop I of human U1 RNA decreased binding of the U1-specific proteins. In contrast, Jones and Guthrie (1990) demonstrated by saturation mutagenesis of the Sm binding site in yeast U5 that function of the Sm site is relatively insensitive to mutation. Our studies on the binding of fibrillarin to the U3 snRNA suggest that Box C is highly sensitive to mutation since a single nucleotide change can diminish binding. Although we have defined the minimum length of the Box C sequence necessary for fibrillarin binding, we have not defined the precise boundaries of the required nucleotides nor have we ruled out the possibility that in other Fb snRNPs a Box C sequence smaller than the nine nucleotide U3 Box C can suffice. Our finding that Box C is sensitive to mutation is supported by elegant studies on the essential elements for U14 snRNA function in yeast (Jarmolowski *et al.*, 1990). Nitrous acid mutations in defective U14 snRNAs are distributed over three domains including Boxes C and D and a third region complementary to 18S rRNA.

The Sm proteins, which bind to a short sequence [PuA(U)$_n$GPu] in spliceosomal snRNAs, are said to be the 'core proteins' of the Sm snRNPs. 'Core' in this case refers to: (i) proteins which form a precursor particle in the absence of an snRNA (Fisher *et al.*, 1985); (ii) proteins which assemble onto the RNA in the cytoplasm (reviewed in Mattaj, 1988); and (iii) proteins which are common to a family of snRNPs. In addition, the Sm proteins play an active role in directing the cap trimethylation and nuclear targeting of the Sm snRNPs. Whether fibrillarin conforms to the definition of a 'core' protein for the Fb snRNPs remains to

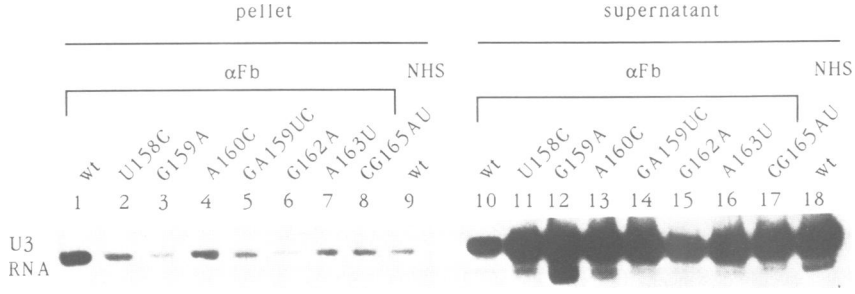

Fig. 5. An intact box C is required for fibrillarin binding to U3. ^{32}pCp-labeled wild-type U3 and seven U3 RNAs with single or double base mutations in Box C (see Table I) were incubated in HeLa WCE for 45 min at 37°C and analyzed for binding to fibrillarin by immunoprecipitation with anti-fibrillarin antibodies (αFb). The pellets (lanes 1−9) and corresponding supernatants (10−18) were analyzed on an 8% denaturing polyacrylamide gel. The binding of the mutated U3 RNAs to fibrillarin (lanes 2−8) was 5−10% of the binding of the U3 RNA (lane 1). Background binding (lane 9) with NHS was ~5% of the binding of U3 RNA with anti-fibrillarin.

be seen. Although it is common to six nucleolar snRNPs, it is not yet known whether it pre-assembles with other proteins to form a precursor particle that then associates with these snRNAs in the cytoplasm. The results reported here are a prerequisite to studies that address the necessity of fibrillarin binding for cap trimethylation of U3, U8 and U13 snRNAs and for the nucleolar localization of all the Fb snRNPs.

Materials and methods

Reagents

Restriction enzymes and DNA modifying enzymes were from Boehringer-Mannheim, New England Biolabs and Pharmacia; *Taq* polymerase from Perkin-Elmer Cetus. Oligonuclotides were synthesized on an Applied Biosystems oligonucleotide synthesizer by Dr John Flory, Yale University School of Medicine.

Antibodies

Sera from patients with scleroderma containing anti-fibrillarin antibodies were obtained from Dr Joseph Craft, Yale University School of Medicine. #1875 and #1746, which are specific for fibrillarin by RNA and protein immunoprecipitation, were used in this study. Normal human serum was generously donated by Scott Seiwert in our laboratory.

Purification of RNAs from HeLa cells

The U3, U8, U13, X and Y RNAs, all of which are indirectly immunoprecipitable by anti-fibrillarin sera, were purified from HeLa cells and ^{32}pCp-labeled according to standard procedures (Tyc and Steitz, 1989). A U3 RNA containing only nucleotides 80-217 (the 3′ end) was created *in vitro* by RNase H-directed digestion of ^{32}pCp-labeled U3 RNA using an oligonucleotide complementary to nucleotides 64-79 (see Figure 1a; Kass *et al.*, 1990). RNAs and U3 RNA fragments were purified on denaturing polyacrylamide gels for use in the assembly assay.

Extract preparation and fibrillarin binding assay

Whole cell extracts (WCE) were prepared from HeLa cells using the procedure of Manley *et al.* (1980). At the final step, the extract was dialyzed into 20 mM HEPES pH 7.9, 50 mM KCl, 0.1 mM EDTA, 2 mM $MgCl_2$, 17% glycerol and 2 mM DTT.

RNA was assembled with fibrillarin in HeLa WCE. Generally, 5000 c.p.m. of purified U3 RNA prepared as outlined above was added to 15 μl of WCE containing 2 μl RNasin. The volume was brought to 25 μl with water, giving a proportion of 60% extract in the reconstitution mixture. The RNA dissolved in water was heated to 90°C and cooled slowly over 15 min before addition. The reaction was incubated at 30°C for 45 min and then the input U3 RNA was assayed for binding to fibrillarin by immunoprecipitation with anti-fibrillarin antibodies.

Immunoprecipitation

Either sera (5 μl) from scleroderma patients containing antibodies to fibrillarin or normal human serum was added to 2.5 mg protein A-Sepharose (PAS) in NET-2 (150 mM NaCl, 50 mM Tris pH 7.5, 0.05% NP-40) and incubated for 1 h at room temperature. The antibody-PAS pellets were washed four times, and then the reconstitution reaction was added to the antibody-PAS pellet in 0.5 ml NET-2 and nutated for 2 h at 4°C. The immunoprecipitates were processed according to standard procedures (Steitz, 1989) and the RNA recovered from both pellets and supernatants analyzed on 8% denaturing polyacrylamide gels. Results were quantified on a Molecular Dynamics Phosphorimager.

Cloning of the human U3 cDNA for in vitro RNA synthesis

The human U3 RNA was cloned behind a T7 polymerase promoter using the polymerase chain reaction (PCR) as follows. Two deoxyoligonucleotides corresponding to the 5′ and 3′ ends of the U3 RNA were synthesized (see Figure 1b). The 3′ oligonucleotide has 44 nucleotides complementary to the U3 RNA 3′ end preceded by an *Rsa*I site (four nucleotides) and a *Sac*I site (six nucleotides). The 5′ oligonucleotide contains 31 nucleotides of U3 sequence preceded by a T7 promoter (17 nucleotides), and an *Eco*RI site (six nucleotides). At their extreme 5′ termini the oligonucleotides end with either six cytidines or six guanosines, respectively, as a 'clamp' to facilitate cleavage by the restriction endonucleases. The *Eco*RI and *Sac*I sites are for cloning the PCR fragment; digestion at the *Rsa*I site gives a U3 RNA with a correct 3′ end upon transcription. Three guanosines were included between the T7 promoter and the *in vivo* U3 RNA sequence to provide a strong trancriptional start site for the T7 polymerase.

U3 RNA was extracted from 1 g of HeLa cells by anti-fibrillarin precipitation (Tyc and Steitz, 1989), gel purified, and used for first strand synthesis. The U3 RNA pellet and 100 pmol 3′ end primer were annealed in a total volume of 5 μl. The RNA-oligonucleotide mixture was heated to 90°C for 2 min and then cooled slowly for 45 min. Reverse transcription was performed in 50 mM Tris pH 8.3, 6 mM $MgCl_2$, 40 mM KCl, 1 mM DTT, 1 mM deoxynucleotides and 13 units reverse transcriptase (Pharmacia) for 1 h at 37°C. One-fourth of this reaction was then used as the template for PCR, performed according to Toczyski and Steitz (1991). Thirty cycles of PCR were carried out on a Perkin-Elmer Cetus thermocycler. The annealing was at 60°C, extension at 72°C and denaturation at 94°C.

The PCR reaction was ethanol precipitated and fractionated on an 8% denaturing polyacrylamide gel. The single correctly-sized band was excised, eluted, and then cut with *Eco*RI and *Sac*I. The cut PCR product was cloned into the *Eco*RI-*Sac*I site of pSP64 (Promega Biotech). The entire insert was sequenced and found to be correct. Run-off trancription after digestion with *Rsa*I gives a 3′ end identical to the *in vivo* U3 RNA but with 3 additional guanosines at the 5′ end.

Site-directed mutagenesis

The *Eco*RI-*Hin*dIII fragment of the U3 pSP64 constuct was subcloned into the *Eco*RI-*Hin*dIII sites of M13 mp18 to generate a single-stranded template for site-directed mutagenesis. Mutagenesis was performed according to the method of Zoller and Smith (1983) as modified by Kunkel (1987). Seven single or double base substitutions in the U3 Box C region and the oligonucleotides that were used in the synthesis are depicted in Table I. Using comparable methods, four Box D mutations (U208C, C209Δ, U210C, G211U) as well as a deletion/substitution mutation of the U3 loop at nucleotides 134-138 (UUGGC to ACACA) were also made (see Figure 1a). Potential mutations were screened by direct DNA sequencing; the mutation rate varied from 10-90%. All clones were sequenced in their entirety. Replicative form DNA was prepared according to standard procedures (Maniatis *et al.*, 1982).

Transcription

To generate full-length U3 RNAs, 5 μg of either plasmid or RF DNA were digested with *Rsa*I. For generation of 3′ end deletions, DNA was cut with *Bst*UI, *Fnu*4HI or *Dde*I (see Figures 1 and 4a). Transcriptions were performed according to Melton *et al.*(1984) using 1 μg of DNA template. No radioactive nucleotides were used. After digestion with DNase the RNA transcript was purified over a G-50 spin column. The RNA was labeled with ^{32}pCp in 50 mM HEPES pH 8.3, 20 mM $MgCl_2$, 3 mM DTT, 400 μM ATP, 40 μCi ^{32}pCp (3000 Ci/mmol) and 70 units of RNA ligase for 1 h at 37°C. The RNA was ethanol precipitated and run on an 8% denaturing polyacrymide gel. RNA was extracted from the gel by standard procedures (Maniatis *et al.*, 1982) and then used in assembly reactions.

Acknowledgements

We thank Michelle Caizergues-Ferrer, Peter Glazer, Gregg Morin, David Toczyski, Kazio Tyc and David Wassarman for helpful discussions, Karen Montzka for the initial HeLa whole cell extracts and Joe Craft for anti-fibrillarin antibodies. We thank David Tollervey for generously sharing results before publication. This research was supported by grant GM26154 from the National Institutes of Health. S.J.B. is a fellow of the Leukemia Society of America.

References

Dignam,J.D., Lebovitz,R.M. and Roeder,R.G. (1983) *Nucleic Acids Res.*, **11**, 1475-1489.

Feeney,R.J., Sauterer,R.A., Feeney,J.L. and Zieve,G.W. (1989) *J. Biol. Chem.*, 264, 5776-5783.

Fischer,U. and Lührmann,R. (1990) *Science*, **249**, 786-790.

Fisher,D.E., Conner,G.E., Reeves,W.H., Wisniewolski,R. and Blobel,G. (1985) *Cell*, **42**, 751-758.

Hamm,J., Darzynkiewicz,E., Tahara,S.M. and Mattaj,I.W. (1990a) *Cell*, **62**, 569-577.

Hamm,J., Dathan,N.A., Scherly,D. and Mattaj,I.W. (1990b) *EMBO J.*, **9**, 1237-1244.

Hamm,J., Kazmaier,M. and Mattaj,I.W. (1987) *EMBO J.*, **6**, 3479-3485.

Hamm,J., van Santen,V.L., Spritz,R.A. and Mattaj,I.W. (1988) *Mol. Cell. Biol.*, **8**, 4787-4791.

Hartshorne,T. and Agabian,N. (1991) *J. Cell. Biochem.*, suppl. **15D**, 67.

Heintz,N. and Roeder,R.G. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 2713-2717.

Henriquez,R., Blobel,G. and Aris,J.P. (1990) *J. Biol. Chem.*, **265**, 2209-2215.

Hernandez,N. and Weiner,A. (1986) *Cell*, **47**, 249–258.
Hughes,J.M.X., Konings,D.A.M. and Cesareni,G. (1987) *EMBO J.*, **6**, 2145–2155.
Jansen,R.P., Hurt,E.C., Kern,H., Lehtonen,H., Carmo-Fonseca,M., Lapeyre,B. and Tollervey,D. (1991) *J. Cell Biol.*, **113**, 715–729.
Jarmolowski,A., Zagorski,J., Li,H. and Fournier,M.J. (1990) *EMBO J.*, **9**, 4503–4509.
Jeppesen,C., Stebbins-Boaz,B. and Gerbi,S.A. (1988) *Nucleic Acids Res.*, **16**, 2127–2148.
Jones,M.H. and Guthrie,C. (1990) *EMBO J.*, **9**, 2555–2561.
Kass,S., Tyc,K., Steitz,J.A. and Sollner-Webb,B. (1990) *Cell*, **60**, 897–908.
Kunkel,T.A., Roberts,J.D. and Zakour,R.A. (1987) *Methods Enzymol.*, **154**, 367–382.
Lapeyre,B., Mariottini,P., Mathieu,C., Ferrer,P., Amaldi,F., Amalric,F. and Caizergues-Ferrer,M. (1990) *Mol. Cell. Biol.*, **10**, 430–434.
Liautard,J.P., Sri-Widada,J., Brunel,C. and Jeanteur,P. (1982) *J. Mol. Biol.*, **162**, 623–643.
Lischwe,M.A., Ochs,R.L., Reddy,R., Cook,R.G., Yeoman,L.C. Tan, E.M., Reichlin,M. and Busch,H. (1985) *J. Biol. Chem.*, **260**, 14304–14310.
Liu,J. and Maxwell,E.S. (1990) *Nucleic Acids Res.*, **18**, 6565–6571.
Lührmann,R. (1988) In Birnstiel,M.L. (ed.), *Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles*. Springer Verlag, Heidelberg, pp. 71–99.
Lutz-Reyermuth,C. and Keene,J.D. (1989) *Mol. Cell. Biol.*, **9**, 2975–2982.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Manley,J.L., Fire,A., Cano,A., Sharp,P.A. and Gefter,M.L. (1980) *Proc. Natl. Acad. Sci. USA*, 77, 3855–3859.
Mattaj,I.W. (1988) In Birnstiel,M.L. (ed.), *Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles*. Springer Verlag, Heidelberg, pp. 100–114.
Melton,D.A., Krieg,P.A., Rebagliati,M.R., Maniatis,T., Zinn,K. and Green,M.R. (1984) *Nucleic Acids Res.*, **12**, 7035–7056.
Nakamura,T., Prestayko,A.N. and Busch,H. (1968) *J. Biol. Chem.*, **243**, 1368–1375.
Neuman de Vegvar,H.E. and Dahlberg,J.E. (1990) *Mol. Cell. Biol.*, **10**, 3365–3375.
Parker,K.A. and Steitz,J.A. (1987) *Mol. Cell. Biol.*, **7**, 2899–2913.
Patton,J.R. and Pederson,T. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 747–751.
Patton,J.R., Patterson,R.J. and Pederson,T. (1987) *Mol. Cell. Biol.*, **7**, 4030–4037.
Prestayko,A.W., Tonato,M. and Busch,H. (1970) *J. Mol. Biol.*, **47**, 505–515.
Query,C.C., Bentley,R.C. and Keene,J.D. (1989) *Mol. Cell. Biol.*, **9**, 4872–4881.
Savino,R. and Gerbi,S.A. (1990) *EMBO J.*, **9**, 2299–2308.
Scherly,D., Boelens,W., van Venrooij,W.J., Dathan,N.A., Hamm,J. and Mattaj,I.W. (1989) *EMBO J.*, **8**, 4163–4170.
Scherly,D., Boelens,W., Dathan,N.A., van Venrooij,W.J. and Mattaj,I.W. (1990) *Nature*, **345**, 502–506.
Schimmang,T., Tollervey,D., Kern,H., Frank,R. and Hurt,E.C. (1989) *EMBO J.*, **8**, 4015–4024.
Spritz,R.A., Strunk,K., Surowy,C.S., Hoch,S.O., Barton,D.E. and Francke,U. (1987) *Nucleic Acids Res.*, **15**, 10373–10391.
Steitz,J.A. (1989) *Methods Enzymol.*, **180**, 468–481.
Surowy,C.S., van Santen,V.L., Scheib-Wixted,S.M. and Spritz,R.A. (1989) *Mol. Cell. Biol.*, **9**, 4179–4186.
Toczyski,D.P.W. and Steitz,J.A. (1991) *EMBO J.*, **10**, 459–466.
Tyc,K. and Steitz,J.A. (1989) *EMBO J.*, **8**, 3113–3119.
Weinberg,R.A. and Penman,S. (1968) *J. Mol. Biol.*, **38**, 289–304.
Wise,J.A. and Weiner,A.M. (1980) *Cell*, **22**, 109–118.
Yuo,C.Y. and Weiner,A.M. (1989) *Genes Dev.*, **3**, 697–707.
Zieve,G.W. and Sauterer,R.A. (1990) *Crit. Rev. Biochem. Mol. Biol.*, **25**, 1–46.
Zoller,M.J. and Smith,M. (1983) *Methods Enzymol.*, **100**, 468–500.

Received on April 19, 1991