

Coiled Bodies without Coilin

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Nuclei assembled *in vitro* in *Xenopus* egg extract contain coiled bodies that have components from three different RNA processing pathways: pre-mRNA splicing, pre-rRNA processing, and histone pre-mRNA 3'-end formation. In addition, they contain SPH-1, the *Xenopus* homologue of p80-coilin, a protein characteristic of coiled bodies. To determine whether coilin is an essential structural component of the coiled body, we removed it from the egg extract by immunoprecipitation. We showed that nuclei with bodies morphologically identical to coiled bodies (at the light microscope level) formed in such coilin-depleted extract. As expected, these bodies did not stain with antibodies against coilin. Moreover, they failed to stain with an antibody against the Sm proteins, although Sm proteins associated with snRNAs were still present in the extract. Staining of the coilin- and Sm-depleted coiled bodies was normal with antibodies against two nucleolar proteins, fibrillarin and nucleolin. Similar results were observed when Sm proteins were depleted from egg extract: staining of the coiled bodies with antibodies against the Sm proteins and coilin was markedly reduced but bright nucleolin and fibrillarin staining remained. These immunodepletion experiments demonstrate an interdependence between coilin and Sm snRNPs and suggest that neither is essential for assembly of nucleolar components in coiled bodies. We propose that coiled bodies are structurally heterogeneous organelles in which the components of the three RNA processing pathways may occur in separate compartments.

INTRODUCTION

Coiled bodies are discrete nuclear organelles ranging in diameter from less than 1 μm in somatic cells to more than 10 μm in oocytes. They are distinct from but often closely associated with nucleoli and with the speckles of somatic nuclei and the B snurposomes of amphibian oocytes (for review, Lamond and Carmo-Fonseca, 1993; Spector, 1993; Bohmann *et al.*, 1995; Gall *et al.*, 1995). They contain a unique protein, p80-coilin, originally characterized from human tissue culture nuclei (Andrade *et al.*, 1991; Raška *et al.*, 1991) and often used as a definitive marker for coiled bodies. SPH-1, the *Xenopus* homologue of coilin, was described from the sphere organelles of the germinal vesicle (GV; Tuma *et al.*, 1993), which are the oocyte counterparts of somatic coiled bodies (Gall *et al.*, 1995; Roth, 1995). Components involved in pre-mRNA splicing, pre-rRNA processing, and histone pre-

mRNA 3'-end formation are also found in coiled bodies of several cell types (Carmo-Fonseca *et al.*, 1991; Raška *et al.*, 1991; Huang and Spector, 1992; Wu and Gall, 1993; Bauer *et al.*, 1994; Jiménez-García *et al.*, 1994; Frey and Matera, 1995). Coiled bodies in somatic cells do not accumulate poly(A) RNA and do not stain with an antibody against SC35, one of the SR group of essential splicing factors (Carmo-Fonseca *et al.*, 1991; Raška *et al.*, 1991; Huang and Spector, 1992), and for these reasons are not thought to be the actual site of pre-mRNA splicing.

The work reported herein was carried out on nuclei assembled *in vitro* in *Xenopus* egg extract. After the initial discovery by Lohka and Masui (1983, 1984) that sperm heads will swell in egg extract to form typical pronuclei, numerous investigators have used this system to study nuclear protein import, nuclear envelope assembly, and DNA replication (for review, Smythe and Newport, 1991; Almouzni and Wolffe, 1993). Nuclei assembled *in vitro* lack typical nucleoli and speckles, but they contain coiled bodies. These coiled bodies

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were first studied by Bell *et al.* (1992), who called them prenucleolar bodies, because they contained four nucleolar proteins: fibrillarin, nucleolin, B23/NO38, and the *Xenopus* 180-kDa nucleolar protein. Subsequently, we demonstrated *Xenopus* coilin (SPH-1) and components of the three RNA processing systems by immunofluorescence and in situ hybridization (Bauer *et al.*, 1994), showing that these structures are, in fact, similar in composition to coiled bodies from other nuclei.

Although very little is known about the function of coilin, its unique association with coiled bodies suggests that it might play an essential role either in the formation or maintenance of these structures. The *Xenopus* egg extract offers a unique system in which to study these possibilities, because various components can be added to or removed from the extract before the addition of sperm heads and the formation of nuclei (for example, Newport *et al.*, 1990; Adachi and Laemmli, 1994; Felix *et al.*, 1994; Saitoh *et al.*, 1996). We show herein that extracts depleted of coilin support the assembly of nuclei that contain structures similar to the coiled bodies of control nuclei. Immunofluorescent staining of these bodies indicates that they no longer contain coilin or Sm proteins but do contain the nucleolar proteins fibrillarin and nucleolin. Similarly, when most of the Sm snRNPs are depleted from the extract, coiled bodies contain reduced amounts of Sm proteins and coilin, whereas the content of fibrillarin and nucleolin remains unchanged. These immunodepletion experiments demonstrate an interdependence between coilin and the Sm snRNPs and suggest that neither is essential for assembly of nucleolar components in coiled bodies.

MATERIALS AND METHODS

Animals and Egg Collection

Adult *Xenopus laevis* stocks purchased from Nasco (Fort Atkinson, WI) were maintained in fiberglass aquaria and fed on beef heart. Frogs were primed with 100 IU of pregnant mares' serum (Sigma, St. Louis, MO) 3–6 days before injection of 300–400 IU of human chorionic gonadotropin (Sigma) into the dorsal lymph sac. Frogs were left overnight to lay their eggs in a 1-gallon glass aquarium containing 2 l of 0.1 M NaCl. The females usually laid between 5 and 50 ml of eggs overnight if left undisturbed in the dark at 19°C.

Nuclear Assembly Extract

Eggs from a single female were dejellied by swirling in three changes of 2% cysteine (pH 7.8) for no more than 10 min. Fresh interphase egg extract was prepared in S-lysis buffer by using the protocol described by Newmeyer and Wilson (1991). Briefly, eggs were gently packed and centrifuged at 4°C for 13 min at 10,000 rpm (9000 × g) in the SW56 rotor (Beckman Instruments, Fullerton, CA), after which the yolk and lipid layers were discarded. In some experiments the crude extract was further fractionated by centrifugation in a Beckman TLS-55 rotor at 55,000 rpm (200,000 × g) for 1 h at 4°C. The supernatant and membrane fractions were removed separately by pipette. The supernate was centrifuged again at 4°C for 20 min at 55,000 rpm to remove residual membranes, after which an ATP regeneration system was added (1 mM ATP, pH 7.0, 10 mM

phosphocreatine, 50 μg/ml creatine phosphokinase). The final supernate was distributed into 30-μl samples and frozen at -70°C. The membranes were washed in 3–4 vol of membrane wash buffer (50 mM KCl, 250 mM sucrose, 2.5 mM MgCl₂, 50 mM HEPES, pH 8.0, 1 mM dithiothreitol, 1 mM ATP, 1 μg/ml leupeptin, and 1 μg/ml aprotinin) and centrifuged in a TLS-55 rotor at 20,000 rpm for 20 min at 4°C. The wash was repeated a second time and the pellet was resuspended in 0.5 M sucrose in membrane wash buffer. The membranes were frozen at -70°C in 7-μl aliquots.

Demembrated *Xenopus* sperm were prepared with lysolecithin and stored at -70°C in small aliquots containing 80,000 sperm heads/μl (Newmeyer and Wilson, 1991). To assemble nuclei in crude extract, 1 μl of sperm was added to 30 μl of crude egg extract and held at room temperature until nuclei were completely swollen (1–1.5 h). Fractionated extract was assembled by adding 7 μl of membranes to 30 μl of supernate, mixing thoroughly, and adding 1 μl of sperm. These nuclei were completely swollen 1.5–2 h after the addition of sperm.

Immunodepletion

It was possible to deplete coilin from crude *Xenopus* egg extract with anti-coilin magnetic beads (Dynal, Great Neck, NY), whereas the viscosity of the extract prevented efficient use of Sepharose beads. A slurry of 50–100 μl of magnetic beads, coated with goat anti-mouse IgG, was washed twice with PBS (125 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 8.1 mM Na₂HPO₄) and then nutated at 4°C with two successive aliquots of 0.5–1 ml of ice-cold bovine serum albumin (BSA, 10 mg/ml), each for 0.5 h. The BSA-blocked beads were then washed four times with ice-cold PBS, separated into two fractions, and nutated overnight at 4°C with either 1 ml of concentrated anti-coilin monoclonal antibody (mAb) H1 (Tuma *et al.*, 1993) or with 1 ml of PBS. mAb H1 consisted of cell culture supernate concentrated ≈10-fold by ultrafiltration (Centriprep-100; Amicon, Beverly, MA). Both sets of beads were washed four or five times with ice-cold XB buffer (100 mM KCl, 1 mM MgCl₂, 0.1 mM CaCl₂, 50 mM sucrose, 10 mM HEPES, pH 7.7). Thirty microliters of crude extract were incubated with 50% of the anti-coilin beads or 50% of the control beads, nutating for 1 h at 4°C. The extracts were removed and added to the second set of ice-cold anti-coilin and control beads, incubating for an additional hour. The coilin-depleted and control (mock-depleted) extracts were removed and 1 μl of demembrated sperm was added to each sample.

For Sm depletion, 20 μl of packed protein A-Sepharose beads (Pharmacia Biotech, Piscataway, NJ) were blocked with 30 μl of high-speed supernate, nutating at 4°C for 1 h. The beads were then washed two times with ice-cold PBS and further blocked with BSA. The rest of the procedure followed as above for the coilin depletion, except that beads were incubated overnight with mAb Y12 (Lerner *et al.*, 1981), either from cell culture supernate or mouse serum. After the depletion, membranes and sperm were added to the Sm-depleted and control samples.

Cytology

For immunofluorescent studies, 2 μl of egg extract were mixed with 2 μl of 4% paraformaldehyde in OR2 buffer (Wallace *et al.*, 1973) in the center of a siliconized 18-mm² coverslip and inverted onto a "subbed" slide (prepared by dipping standard 3 × 1-inch microscope slides in 0.5% gelatin, 0.05% CrK(SO₄)₂·12H₂O, drying in air, and baking 1 h at 65°C). The preparation was then squashed by inverting the slide onto a piece of filter paper and pressing on the coverslip region without moving the slide. The squashed preparation was placed in liquid N₂ until frozen, the coverslip was flipped off with a razor blade, the preparation was placed in 95% ethanol for 1 to 2 h, and then immediately stained. We found that prefixed squashed preparations gave the brightest immunofluorescent signal with all antibodies used in this study.

Immunofluorescence and Antibodies

Slides that had been prefixed, frozen, and held in 95% ethanol for 1 to 2 h were hydrated through a descending alcohol series, rinsed in PBS, and blocked with 10% horse serum in PBS for 30 min. The block was removed and the primary antibody was added for 1 h. Slides were then rinsed in 10% horse serum and the secondary antibody was incubated for 1 h. The slides were rinsed again in 10% horse serum and mounted in 50% glycerol containing 1 mg/ml phenylenediamine (pH 9) and 0.2 $\mu\text{g}/\text{ml}$ 4',6-diamidino-2-phenylindole. When not being observed, slides were stored at -20°C . The secondary antibodies were affinity-purified rhodamine- or fluorescein-labeled goat anti-mouse or goat anti-rabbit IgG (Cappel/Organon Teknika, Durham, NC). The following primary antibodies were used: mAb H1 against *Xenopus* coilin (Tuma *et al.*, 1993), mAb Y12 against the Sm epitope of snRNPs (Lerner *et al.*, 1981), mAb 17C12 against fibrillarin (Hultman *et al.*, 1994), rabbit serum R2D2 against *Xenopus* nucleolin (Heine *et al.*, 1993), and mAb anti-SC35 against human SR protein SC35 (Fu and Maniatis, 1990).

Western and Northern Blotting

Various amounts of egg extract were mixed with PBS to bring them to a final volume of 10 μl ; bead samples were prepared by removing all buffer and adding 5–10 μl of PBS. All samples were boiled with the appropriate amount of 2 \times gel buffer for 5 to 10 min and electrophoresed on a 10% polyacrylamide-SDS gel (Laemmli, 1970). Proteins on the gel were electroeluted to Immobilon membranes (Millipore, Bedford, MA). Western blotting was performed with mAb H1 and detected by chemiluminescence (ECL kit; Amersham Life Science, Arlington Heights, IL).

RNA samples were extracted with phenol, phenol:chloroform (1:1), and chloroform; precipitated with 0.3 M sodium acetate in 70% ethanol with 5 μg of glycogen as carrier; and electrophoresed on 8 M urea-10% acrylamide gels. RNA was transferred to nylon Gene-Screen filters (New England Nuclear, Boston, MA) by electroblotting and was hybridized with ^{32}P -labeled antisense probes against various snRNAs. Clones used for the synthesis of ^{32}P -labeled riboprobes were as follows: human U1 and U2 snRNAs (Wu *et al.*, 1991); human U4, U5, and U6 snRNAs (Black and Pinto, 1989); and *Xenopus* U7 snRNA (Wu and Gall, 1993).

RESULTS

Immunodepletion of Coilin from Crude Egg Extract

The *Xenopus* homologue of mammalian p80-coilin, SPH-1, was originally described from the GV of oocytes, where it was detected with a specific antibody, mAb H1 (Tuma *et al.*, 1993). At the time of GV breakdown, coilin is released into the cytoplasm and can be demonstrated in immunoblots of egg extract (Figure 1A, lane 6). When demembrated sperm are incubated in egg extract, the newly assembled nuclei import coilin and concentrate it in the coiled bodies (Bauer *et al.*, 1994). In this study our initial aim was to determine what effect the depletion of coilin from the egg extract would have on the formation of nuclei and coiled bodies. To accomplish this, we incubated crude *Xenopus* egg extract with mAb H1 attached to magnetic beads. Western blots showed that coilin was readily detectable in control (mock-depleted) extract but not in extract treated with mAb H1 (Figure 1A, lanes 3 and 6). Although two successive batches of beads were routinely used for depletion, almost all

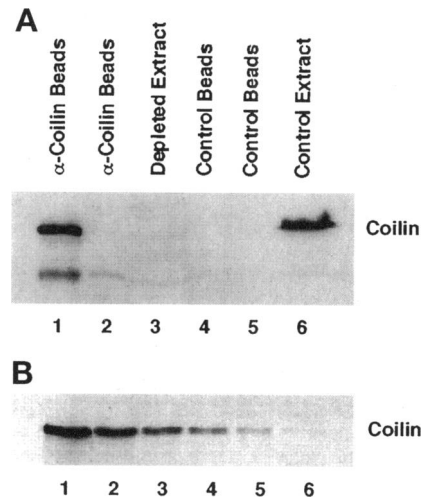


Figure 1. (A) Depletion of coilin from *Xenopus* egg extract. Western blot of bead and extract fractions detected with mAb H1 against SPH-1 (*Xenopus* coilin; Tuma *et al.*, 1993). Lanes 1 and 2 show material recovered from the first and second sets of mAb H1-coated magnetic beads used to immunoprecipitate coilin from 30 μl of crude extract. Coilin (M_r -80 kDa) and a presumed breakdown product (M_r -50 kDa) are evident on the first set of beads (lane 1) but barely so on the second (lane 2). Coilin is almost undetectable in 6 μl of depleted extract (lane 3), whereas it is readily detectable in 6 μl of mock-depleted extract (lane 6). No coilin is immunoprecipitated by either the first or second set of control beads (lanes 4 and 5). (B) Western blot of serial dilutions of crude extract detected with mAb H1. Lanes 1–6 contain 5.0 μl , 2.5 μl , 1.3 μl , 0.6 μl , 0.3 μl , and 0.2 μl of extract, respectively. Coilin is barely detectable in 0.2 μl of crude egg extract, demonstrating that the depletion of coilin in A is 97% or better.

detectable coilin was, in fact, removed from the extract by the first batch (Figure 1A, lanes 1 and 2). As expected, no coilin was found on control beads that lacked antibody (Figure 1A, lanes 4 and 5). A comparison of the depleted extract with serial dilutions of control extract showed that depletion was 97% or better (Figure 1B).

When demembrated sperm were incubated in coilin-depleted extract, morphologically typical nuclei were assembled as rapidly as in mock-depleted extract, although in both cases assembly was somewhat slower than in extract that had not been subjected to any manipulation. These nuclei contained structures whose number, size, and general appearance by phase-contrast microscopy were similar to the coiled bodies in control nuclei (Figure 2, B, D, F, and H). However, these structures were completely unstained by mAb H1 against *Xenopus* coilin, in contrast to the brightly stained coiled bodies in nuclei assembled in mock-depleted extract (Figure 2, A and C). For simplicity, we will refer to these structures as coiled bodies throughout this article, despite their lack of coilin.

Unexpectedly, coiled bodies formed in depleted extract failed to stain with mAb Y12, which recognizes

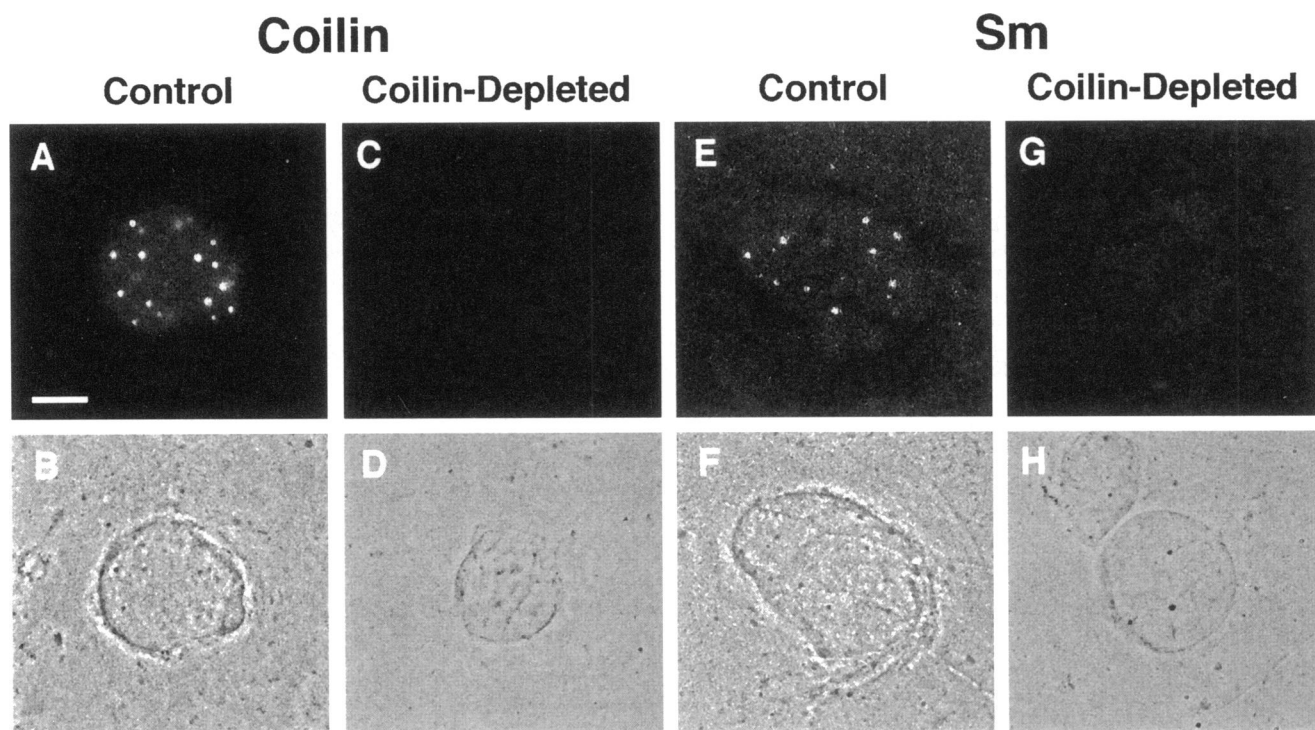


Figure 2. Pronuclei assembled *in vitro* from *Xenopus* sperm heads after incubation in control (mock-depleted) or coilin-depleted crude *Xenopus* egg extract. (A, C, E, and G) Immunofluorescent images. (B, D, F, and H) Phase-contrast images of the same nuclei showing coiled bodies in both control and coilin-depleted samples. (A and C) Nuclei stained with mAb H1 against *Xenopus* coilin (Tuma *et al.*, 1993). (E and G) Nuclei stained with mAb Y12 against the Sm proteins (Lerner *et al.*, 1981). Coiled bodies assembled in coilin-depleted extract failed to stain with antibodies against either coilin or the Sm proteins. Bar, 10 μ m.

the Sm proteins associated with the splicing snRNPs (Lerner *et al.*, 1981), whereas they normally stain quite brightly (Figure 2, E and G). However, they did stain normally with mAb 17C12 against fibrillarin (Hultman *et al.*, 1994; Figure 3, A and C) and rabbit serum R2D2 against *Xenopus* nucleolin (Heine *et al.*, 1993; Figure 3, E and G), suggesting that coilin is not required for the incorporation of these nucleolar proteins into coiled bodies.

Northern blots showed that typical levels of the splicing snRNAs U1, U2, U4, U5, and U6 were present in egg extract after coilin had been completely removed (Figure 4A, lanes 1 and 4). Likewise, U7, the minor Sm snRNA involved in histone pre-mRNA 3'-end processing (Birnstiel and Schaufele, 1988), was present at a normal level in coilin-depleted extract (Figure 4B, lanes 1 and 4). Furthermore, none of these snRNAs was found on the antibody-coated beads used to immunodeplete coilin (Figure 4, A and B, lanes 5 and 6). Thus, the failure of coiled bodies from coilin-depleted extract to stain with the anti-Sm antibody was not due to the absence of Sm snRNPs from the egg extract during assembly. These results suggest that coilin may be required either for the import of Sm snRNPs into the nucleus or for their targeting to coiled bodies after import.

Immunodepletion of Sm snRNPs from Fractionated Egg Extract

Since Sm proteins were undetectable in coiled bodies assembled in coilin-depleted extracts, we wanted to determine what components were present in coiled bodies formed in the absence of Sm snRNPs. Because coilin could be removed from crude egg extract by using magnetic beads, we tried to deplete the Sm snRNPs by using the same method. Magnetic beads coated with either goat anti-mouse IgG or rat anti-mouse IgG2a were incubated with mAb Y12. These beads failed to remove a significant portion of Sm snRNPs from crude egg extract or from a high-speed supernatant fraction of extract. We do not know whether mAb Y12 failed to adhere to these beads or binding of the antibody to the beads in some way inhibited interaction with the Sm proteins.

We then tried protein A-Sepharose beads, which have been used routinely to immunoprecipitate Sm snRNPs (Steitz, 1989). Because Sepharose beads were very difficult to remove completely from the highly viscous and heterogeneous crude extract, it was first necessary to fractionate the extract into membrane and supernatant fractions by high-speed centrifugation (Newmeyer and Wilson, 1991). Sepharose beads can

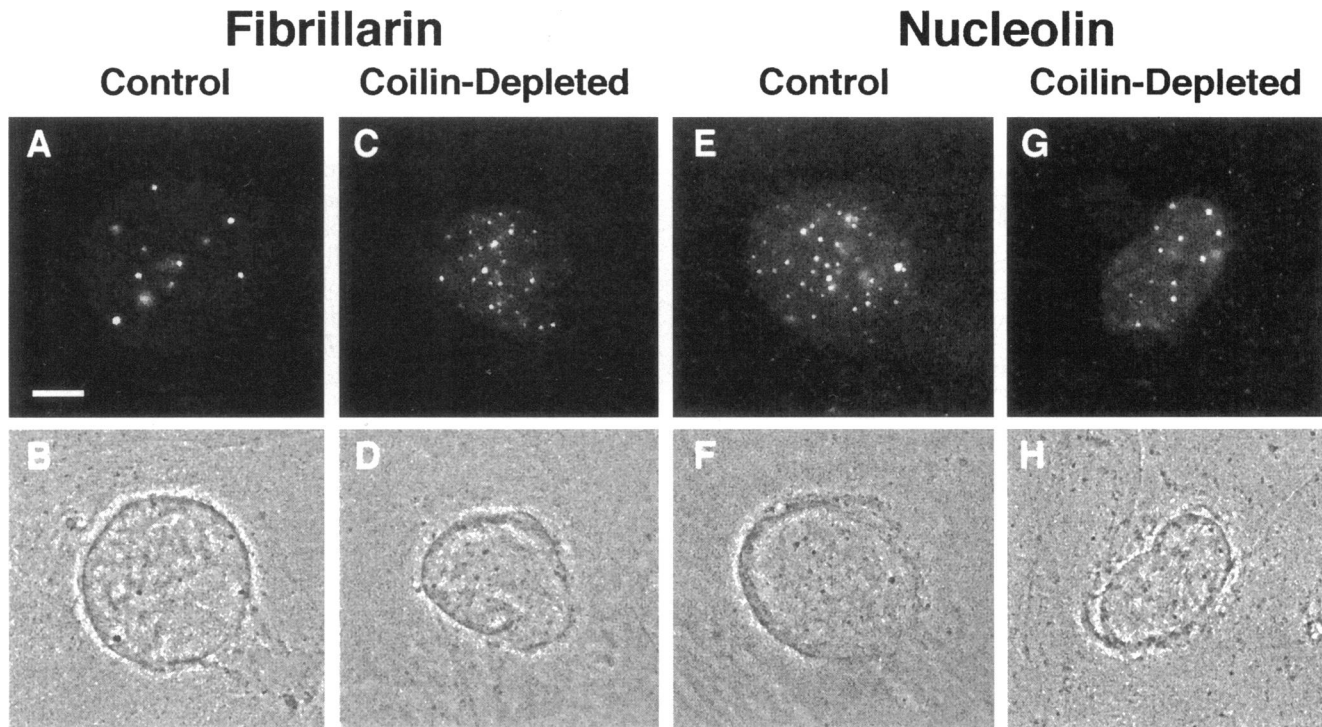


Figure 3. Pronuclei assembled in vitro from *Xenopus* sperm heads after incubation in control (mock-depleted) or coilin-depleted crude *Xenopus* egg extract. (A, C, E, and G) Immunofluorescent images. (B, D, F, and H) Phase-contrast images of the same nuclei. (A and C) Nuclei stained with mAb 17C12 against fibrillarlin (Hultman *et al.*, 1994). (E and G) Nuclei stained with rabbit serum R2D2 against *Xenopus* nucleolin (Heine *et al.*, 1993). Coiled bodies assembled in coilin-depleted extract stained as brightly as those in control extract. Bar, 10 μ m.

be removed from the supernatant fraction by centrifugation. Neither the membrane nor supernatant fraction alone supports nuclear assembly, but they can be recombined to give a functional system. Our protocol involved treating the high-speed supernatant fraction with mAb Y12 attached to protein A-Sepharose beads, removing the beads by centrifugation, adding an appropriate aliquot of membrane fraction, and then adding sperm nuclei. *Xenopus* oocytes and eggs contain an excess of Sm proteins that are not associated with snRNAs (Forbes *et al.*, 1983; Zeller *et al.*, 1983). We assayed for snRNAs, not Sm proteins, for two reasons. First, we assume that only Sm proteins that are actually complexed with snRNAs will enter the nucleus and be targeted to the coiled bodies. Second, although mAb Y12 efficiently immunoprecipitates Sm snRNPs from solution, it gives variable results when used on Western blots. Thus, Northern blots of U1, U2, U4, U5, and U6 snRNAs should give a better measure of the removal of Sm snRNPs from the extract.

We showed that the membrane fraction contained only trace amounts of splicing snRNAs (Figure 5A). Consequently, very few splicing snRNAs (and presumably their associated Sm proteins) were added to the depleted supernate when the two fractions were combined. Northern blots showed that a major frac-

tion of the splicing snRNAs could be removed from the high-speed supernatant by mAb Y12. However, despite the large amount of splicing snRNAs present on the two sets of anti-Sm beads used for depletion, approximately 10% of the total snRNAs remained in the extract (Figure 5B, lanes 2, 4 and 6). It was not feasible to carry out further rounds of depletion, because excessive manipulation of the extract prior to adding the sperm heads led to inefficient assembly of nuclei. As expected, splicing snRNAs were not removed from the extract by control beads (Figure 5B, lanes 1, 3, and 5).

Typical nuclei with coiled bodies were assembled when either snRNP-depleted or mock-depleted supernates were combined with membranes, and demembrated sperm were added (Figure 6, B, D, F, and H). Immunofluorescent staining with mAb Y12 showed a striking reduction but not complete absence of staining in coiled bodies assembled in the snRNP-depleted extract (Figure 6, A and C). Similarly, staining with mAb H1 against coilin was markedly reduced, although not entirely eliminated (Figure 6, E and G). In contrast, coiled bodies assembled in the depleted extract were stained with anti-fibrillarlin or anti-nucleolin antibodies as strongly as those from mock-depleted extract. These results are quite similar to those ob-

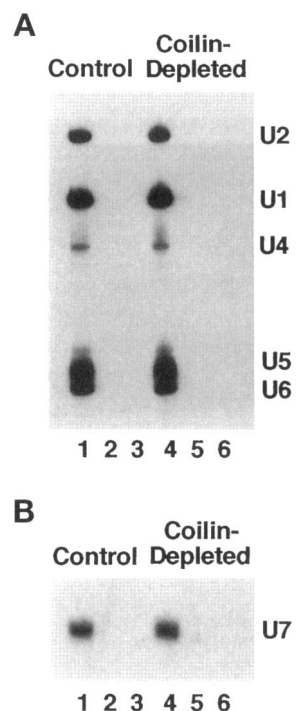


Figure 4. (A) Splicing snRNAs are not removed from *Xenopus* egg extract by immunoprecipitation with mAb H1 against SPH-1 (*Xenopus* coilin; Tuma *et al.*, 1993). Northern blot of bead and extract fractions probed with ^{32}P -labeled antisense RNAs against U1, U2, U4, U5, and U6 snRNAs. Splicing snRNAs are present at the same level in 6 μl of mock-depleted extract (lane 1) and coilin-depleted extract (lane 4). No splicing snRNAs are recovered from control beads (lanes 2 and 3) or mAb H1-coated beads used to immunoprecipitate coilin from egg extract (lanes 5 and 6). (B) Northern blot showing that U7 snRNA is not removed by immunoprecipitation of coilin from egg extract. The blot was probed with ^{32}P -labeled antisense RNA against U7. Lanes are as in A.

tained with coilin-depleted extracts; namely, failure of Sm proteins and coilin to appear in the coiled bodies, with no apparent effect on two nucleolar proteins. The Sm and coilin immunodepletion experiments suggest that coilin and the Sm snRNPs must interact in some way during import into the nucleus or targeting to the coiled bodies.

It has recently been demonstrated that some coilin is immunoprecipitated from *Xenopus* GV extract by mAb Y12 (Tuma, personal communication). In preliminary experiments, we were able to confirm this observation by showing that mAb H1 reacts with material brought down by Y12-coated beads. However, Western blotting shows that our standard Sm-depleted extracts still contain almost as much coilin as control extracts. Therefore, the reduced coilin staining of coiled bodies formed in Sm-depleted extract (Figure 6G) was not due simply to a lack of coilin in the extract.

Thus, the Sm and coilin immunodepletion experiments suggest that coilin and one or more of the Sm snRNPs must interact during their import into the nucleus and/or their targeting to the coiled bodies. At the same time, neither appears to be necessary for proper assembly of two nucleolar components, fibrillarin and nucleolin.

Addition of Coilin to Coilin-depleted Extract

To assess the specificity of the immunodepletions, it would be desirable to add back the component(s) removed by the antibody and show recovery of the

staining characteristics of the coiled bodies. This should be feasible in the case of mAb H1, which is presumably specific for *Xenopus* coilin (SPH-1), but would be very difficult in the case of mAb Y12, which is known to immunoprecipitate all of the splicing snRNPs (Lerner and Steitz, 1979) as well as the U7 snRNP (Birnstiel and Schaufele, 1988). We have carried out a variety of add-back experiments with coilin with the unexpected result that coilin itself seems to inhibit the nuclear assembly reaction. The most clear-cut experiments involved full-length *Xenopus* coilin (SPH-1) expressed in *Escherichia coli* in the pRSET-6 histidine expression vector (Invitrogen, San Diego, CA). Large amounts of highly purified soluble 6-his-coilin were produced with this system by Zheng'an Wu and Michel Bellini, who kindly provided the protein for these experiments. When less than 100 ng of 6-his-coilin was added to a standard 30- μl sample of coilin-depleted extract, nuclei with coiled bodies were formed as usual. However, the coiled bodies did not stain with mAb H1, suggesting that not enough coilin

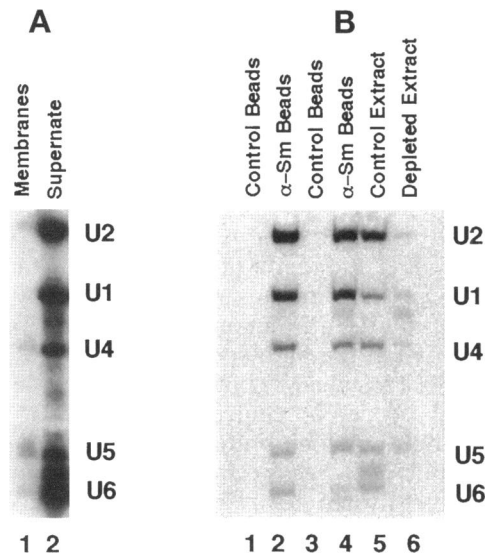


Figure 5. (A) Splicing snRNPs are predominantly in the supernatant after fractionation of *Xenopus* egg extract into membrane and high-speed supernatant fractions. Northern blot probed with ^{32}P -labeled antisense RNAs against U1, U2, U4, U5, and U6 snRNAs. Seven microliters of membrane fraction (lane 1) and 30 μl of high-speed supernatant (lane 2) were compared because these are the amounts used to reconstitute an active extract. (B) Most but not all splicing snRNAs are removed from *Xenopus* egg extract by two successive immunoprecipitations with mAb Y12 against the Sm epitope. Northern blot was probed with ^{32}P -labeled antisense RNAs against U1, U2, U4, U5, and U6 snRNAs. Lanes 5 and 6 show the splicing snRNAs in 6 μl of mock-depleted or Sm-depleted extract. Splicing snRNAs were readily detectable on both sets of mAb Y12-coated Sepharose beads used to immunoprecipitate Sm proteins from 30 μl of supernatant (lanes 2 and 4). No splicing snRNAs were immunoprecipitated by control beads that lacked antibody (lanes 1 and 3).

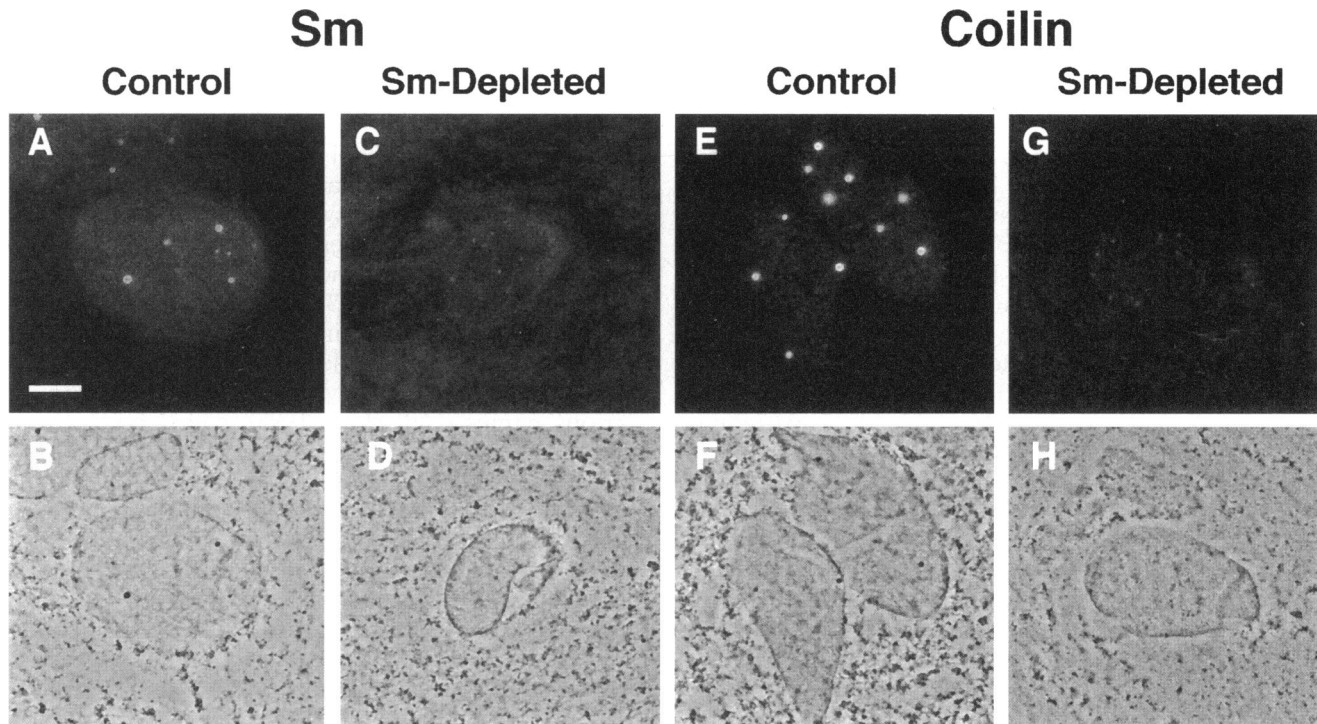


Figure 6. Coiled bodies assembled in Sm-depleted *Xenopus* egg extract are deficient for both Sm proteins and coilin. Pronuclei assembled *in vitro* from *Xenopus* sperm nuclei after incubation in control (mock-depleted) or Sm-depleted *Xenopus* egg extract. (A, C, E, and G) Immunofluorescent images. (B, D, F, and H) Phase-contrast images of the same nuclei. mAb Y12 against the Sm proteins (Lerner *et al.*, 1981) and mAb H1 against *Xenopus* coilin (Tuma *et al.*, 1993) were used to stain nuclei assembled in mock-depleted (A and E) or Sm-depleted extract (C and G). Coiled bodies assembled in Sm-depleted extract were stained much less intensely than those in control extract. Bar, 10 μ m.

had been added. Yet when more protein was added (250–500 ng), thus restoring the amount of coilin in the extract to roughly its original level, nuclei failed to assemble altogether. We also allowed sperm nuclei to incubate in the extract for 10 to 30 min before adding 6-his-coilin, but further swelling was inhibited upon addition of the protein. The same inhibitory result was obtained when more than 100 ng of 6-his-coilin were added to control nondepleted extract, suggesting that the purified protein or some contaminant from *E. coli* was toxic to the nuclear assembly reaction. One possibility is that the state of endogenous coilin differs from that of the coilin expressed in *E. coli*. For instance, the purified protein is probably not phosphorylated. It is not known whether endogenous SPH-1 is phosphorylated, but human p80-coilin is a phosphoprotein (Carmo-Fonseca *et al.*, 1993). Another possibility is that coilin in the extract is complexed with one or more additional proteins that are immunoprecipitated with it by mAb H1. These additional proteins might protect against the toxic effect of pure coilin during nuclear assembly. To assess this latter possibility, we also added back material eluted from the mAb H1-coated beads used for immunodepletion. We showed by Western blotting that the amount of affinity-puri-

fied coilin added back was roughly comparable to that originally present. Again we failed to see mAb H1 staining of coiled bodies in the nuclei that formed. And again, when we increased the amount of coilin to still higher levels, the nuclear assembly reaction itself was inhibited. In future experiments, we will express fragments of coilin as fusion proteins in *E. coli* to see whether they will function in the nuclear assembly reaction. Because the first 102 amino acids of p80-coilin are sufficient for targeting to coiled bodies (spheres) in the *Xenopus* GV (Wu *et al.*, 1994a), this fragment will be particularly interesting to study in the egg extract system.

DISCUSSION

Coiled bodies were first observed in the nuclei of vertebrate neurons by Ramón y Cajal (1903), who called them *accessory bodies* because of their frequent association with the nucleolus. They were independently discovered in an electron microscopic study of mammalian nuclei by Monneron and Bernhard (1969), who suggested the name *coiled bodies*, based on their fine structure. A crucial advance in the understanding of coiled bodies was the discovery of p80-coilin and

the cloning of its cDNA (Andrade *et al.*, 1991; Raška *et al.*, 1991; Andrade *et al.*, 1993). Antibodies against coilin made possible the rapid identification of coiled bodies by fluorescence microscopy and the discovery of new components, including a variety of RNA processing snRNPs (for review, Bohmann *et al.*, 1995; Gall *et al.*, 1995). Whereas coilin is quite limited in its distribution, many of these new components are found elsewhere in the nucleus, particularly in the nuclear speckles and the nucleolus. For this reason, coilin has often been used as the defining characteristic of coiled bodies.

Our study was undertaken to determine whether coilin is an essential structural component of coiled bodies. The *Xenopus* egg extract has several unique advantages for this work: 1) numerous pronuclei assemble rapidly, when demembrated sperm heads are added to the extract; 2) these pronuclei have cytologically prominent coiled bodies (Bauer *et al.*, 1994); and 3) components of the extract can be removed or altered before the sperm are added (for review, Newport *et al.*, 1990; Adachi and Laemmli, 1994; Felix *et al.*, 1994; Saitoh *et al.*, 1996). We showed that coilin can be removed from the extract by immunoprecipitation, with no obvious effect on the morphology of the nuclei and coiled bodies subsequently assembled. In this limited sense, coilin is not required for the formation of coiled bodies. However, the situation is clearly more complex. The elimination of coilin led to the failure of the Sm proteins to be included in the coiled bodies, although two nucleolar proteins, fibrillarin and nucleolin, were unaffected. Similarly, when the majority of the Sm snRNPs were removed from the extract, coilin was markedly reduced in the coiled bodies, but again fibrillarin and nucleolin were apparently unaffected.

These results suggest an important interdependence between coilin and the Sm snRNPs, whose nature is at present unclear. One possibility is that coilin and the snRNPs must form a complex before they can be imported into the nucleus. However, immunoprecipitation of coilin does not remove splicing snRNAs or U7 from the extract, as it might if coilin and the Sm snRNPs were tightly associated before import into the nucleus. Alternatively, coilin and the Sm snRNPs may enter the nucleus independently, but must associate prior to targeting to the coiled bodies. At present, there is no simple way to cleanly separate *in vitro* assembled nuclei from the extract in which they are formed, and so it has not been possible to use biochemical techniques to assess the state of coilin and the Sm snRNPs in these nuclei.

The present experiments bear on the question of what criteria should be used in defining coiled bodies in different cell types and different organisms. Although the original fine-structure criterion (Monneron and Bernhard, 1969) has been extremely useful, it is

not obvious that all bodies with similar composition or that carry out similar functions will exhibit precisely the same fine structure. For example, at the electron microscopic level, the structures assembled *in vitro* in egg extract appear uniformly electron dense, unlike typical coiled bodies (Bell *et al.*, 1992), although their composition is very similar to that of coiled bodies in tissue culture nuclei, as we showed earlier (Bauer *et al.*, 1994). With the discovery of p80-coilin in mammals (Andrade *et al.*, 1991) and its homologue SPH-1 in *Xenopus* (Tuma *et al.*, 1993), the tendency has been to use coilin itself as the defining criterion of coiled bodies. This definition would encompass at least four structures that have previously been known under various names (Gall *et al.*, 1995): coiled bodies from somatic nuclei (especially tissue culture cells), spheres or sphere organelles of the amphibian GV, Binnenkörper of the insect GV, and the bodies described herein from *in vitro*-assembled pronuclei. In addition to coilin, these organelles all contain RNA processing components, but there are significant differences. The sphere organelle of the amphibian GV is particularly important in the present context, because it contains coilin and the Sm snRNPs (the U7 snRNP as well as the splicing snRNPs) but lacks nucleolar components (fibrillarin, nucleolin, and the snoRNAs U3 and U8; Wu *et al.*, 1994a; Gall *et al.*, 1995). The sphere organelle shows that coilin and the Sm snRNPs can exist together independently of nucleolar components. Conversely, the coilin-depleted bodies described herein illustrate the opposite situation—nucleolar components without coilin or the Sm snRNPs.

The coilin-depleted bodies resemble another well known nuclear organelle, the prenucleolar body, found transiently at the end of mitosis during reformation of the nucleoli. Prenucleolar bodies contain several nucleolar components, including fibrillarin, nucleolin, B23 (NO38), and at least one of the snoRNAs (U3). They are prenucleolar in the sense that they fuse together at the rDNA loci during assembly of the definitive nucleoli. However, they do not themselves contain rDNA and neither synthesize nor contain rRNA (Ochs *et al.*, 1985; Jiménez-García *et al.*, 1989; Azum-Gélade *et al.*, 1994; Jiménez-García *et al.*, 1994). Bell *et al.* (1992) clearly recognized that most of these characteristics apply to the nuclear bodies assembled *in vitro* in egg extract (except that they do not form a nucleolus), and for this reason called them prenucleolar bodies. Our subsequent analysis suggested that their composition more closely resembled that of coiled bodies, because in addition to nucleolar components, they contained the splicing snRNPs, the U7 snRNP, and especially coilin. Quite apart from terminological questions, it appears that coiled bodies, either in egg extract or in tissue culture nuclei, combine the major components of prenucleolar bodies with those of amphibian spheres. Furthermore, within the

amphibian sphere, coilin and the U7 snRNP occur together in the matrix, whereas the splicing snRNPs are limited to sharply defined inclusions and to the similar B snurposomes on the surface (Gall *et al.*, 1995). We suggest that coiled bodies (defined by the presence of coilin) are essentially aggregate structures in which components of the three RNA processing systems may be spatially separate and may vary in their relative amounts in different cell types or under different physiological conditions. The experiments reported herein support this hypothesis by showing that nucleolar components can assemble in the absence of coilin and the splicing snRNPs.

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