Abstract. Sphere organelles are nuclear structures in amphibian oocytes that are easily visible by light microscopy. These structures are up to 10 μm in diameter and have been described morphologically for decades, yet their function remains obscure. The present study defines a protein component of the sphere organelle, named SPH-1, which is recognized by a mAb raised against purified *Xenopus laevis* oocyte nucleoplasm. SPH-1 is an 80-kD protein which is localized specifically to spheres and is undetectable elsewhere on lampbrush chromosomes or in nucleoli. We show using confocal microscopy that SPH-1 is localized to the cortex of sphere organelles. Furthermore, we have isolated a cDNA that can encode SPH-1. When epitope-tagged forms of SPH-1 are expressed in *X. laevis* oocytes the protein specifically localizes to spheres, demonstrating that the cloned cDNA encodes the sphere antigen. Comparison of the predicted amino acid sequence with sequence databases shows SPH-1 is related to p80-coilin, a protein associated with coiled bodies; coiled bodies are nuclear structures found in plant and animal cells. The sphere-specific mAb stains *X. laevis* tissue culture cells in a punctate nuclear pattern, showing that spheres or sphere antigens are present in somatic cells as well as germ cells and suggesting a general and essential function for spheres in all nuclei.

The nuclei of amphibian oocytes contain several large, morphologically distinct structures, including lampbrush chromosomes, nucleoli and "spheres." Sphere organelles were first identified as "knobs" attached to lampbrush chromosomes of the newt *Notophthalmus viridescens* (Gall, 1954; reviewed in Callan, 1986). The term organelle is used to describe spheres because they are distinct structures and larger than some whole cells, yet they are not membrane bound. Structures with similar morphology have since been observed on lampbrush chromosomes of many different amphibian species and in oocyte nuclei of several invertebrates, suggesting that spheres may be common to all animal oocyte nuclei where they perform an unknown yet essential function (Gall and Callan, 1989). In amphibians some spheres appear attached to specific loci on lampbrush chromosomes while others are free in the nucleoplasm. In *Xenopus laevis* there are three chromosomal sphere loci, located on chromosomes VIII, IX, and XVI (Callan et al., 1987), as well as 40 to 60 free spheres (Fig. 1). Free and attached spheres are morphologically indistinguishable. They contain a central electron dense core and a surrounding cortex that is less dense (Kezer et al., 1980). Larger spheres have often been observed with 2 to 3 μm hemispherical masses on their surfaces. These hemispherical masses, which are morphologically distinct from spheres, were recently named "B snurposomes" when antibody staining experiments showed they contain a variety of proteins and small nuclear RNAs involved in pre-mRNA splicing (Wu et al., 1991). Similar studies have also shown that spheres can be stained with two antibodies that bind snRNPs and have, as a result, been referred to as "C snurposomes" (Gall and Callan, 1989; Wu et al., 1991). These common splicing components are associated with nearly all active sites of transcription on lampbrush chromosomes (Gall and Callan, 1989; Wu et al., 1991).

We report here the generation of a mAb that binds exclusively to spheres. We have used this antibody to isolate a cDNA which encodes a sphere-specific protein, SPH-1. The primary amino acid sequence of this sphere protein is related to p80-coilin; p80-coilin is localized to a functionally undefined structure called the "coiled body" found in the nuclei of many different plant and animal cells (Andrade et al., 1991). The similarity between SPH-1 and p80-coilin suggests that spheres may be related to coiled bodies.

Materials and Methods

Isolation of Oocyte Nuclei

Ovary (30 g) was removed from albino frogs and minced with scissors into pieces containing ~30 mature oocytes. The ovary was then divided into two 2.5-150 tissue culture flasks each containing 0.1% collagenase w/v/15 grams ovary in 50 ml of Barth's medium (10 mM Hepes, pH 7.4, 88 mM NaCl, 1 mM KCl, 2.3 mM NaHCO3, 0.82 mM MgSO4, 0.66 mM NaNO3, 0.41 mM CaCl2), and rotated at 75 rpm for 90-120 min at room temperature. Oocytes were sieved from the collagenase using 100 μm nitex. The large oocytes were separated from the smaller oocytes by sequential sieving on...
of as many as 25,000 nuclei have been recovered in one day. The percentage recovery of nuclei from oocytes was determined to be at least 80% and the contents transferred using a large bore pasteur pipette to a 150-ram petri dish containing 50 ml of buffer (10 mM Heps, pH 7.4, 5 mM MgCl₂, 60 mM KCl, 1 mM DTT, 5% glycerol) with a piece of 750 μm nitex in the bottom; the 150-mm dish was placed in a Tupperware dish containing a small amount of wet ice. A 100-mm petri dish was then used to break the oocytes by gently crushing them in the larger dish. Using a dissecting microscope the nuclei were observed as they emerged from the oocytes. The lysate was stored for 10-20 min in a refrigerator to allow the yolk to settle, after which time the dishes were gently swirled to bring the nuclei or germinal vesicles to the center of the dish. Swirling involved picking up the dish and rocking it in a circular motion so that “one edge” was always in contact with the ice-containing dish. Some practice was required to know how vigorously to swirl the dish because yolk obscures the ability to see the nuclei. Once the nuclei are in the center of the dish they are removed to a second dish and reswirled; the swirling was repeated five to six times until the nuclei were separated from the cytoplasmic contents. Nuclei were then transferred to a new dish. The nuclear envelopes adhered to the clean plastic and the dish was turned quickly to create a shear force sufficient to separate the congealed nucleolus from the nuclear envelopes. The percentage recovery of nuclei from oocytes was determined to be at least 80% and the contents of as many as 25,000 nuclei have been recovered in one day.

Monoclonal Antibody Isolation and Characterization

Several mice (RBF/Dn, Jackson Laboratories, Bar Harbor, MN) were each immunized with ~10,000 nuclei over a 6 mo period. Polyclonal antisera were tested by indirect immunofluorescent staining of chromosome preparations (Roth and Gall, 1987; Callan et al., 1987). Hybridoma cell lines were generated by fusing the spleen cells of one of these mice to Fox-NY cells (Taggert and Samloff, 1983), and the supernatants were used to test for indirect immunofluorescent staining of spheres. Hybridoma cell lines that produced sphere binding antibodies were isolated as mAbs producing cell lines by limiting dilution cloning (Harlow and Lane, 1988). Immunoblotting and indirect immunofluorescent staining were done according to published methods (Roth and Gall, 1987; Roth et al., 1991).

A X. laevis young ovary polyA⁺ cDNA expression library made in the vector Lambda Zap (Stratagene Corp., Burlingame, CA) was screened using mAbH1 (Short et al., 1988). Two independent classes of inserts (700 and 1,900 bp) were isolated from the expression library and subcloned into Bluescript plasmids (Stratagene Corp.). Sequencing of the cDNAs revealed an open reading frame that could encode a 43-kD protein and that the 700-bp insert is identical to an internal region of the 1,900-bp clone. Because the 1,900-bp insert appeared to be lacking the 5' end of the open reading frame (ORF), the 700 bp cDNA was used as a hybridization probe to screen a lambda gt10 library of Xenopus ovary cDNAs to obtain a full-length cDNA (Rebagliati et al., 1985; Maniatis et al., 1982). 15 positive phage were isolated from ~1.2 × 10⁶ phage screened. The largest insert, referred to here as cSPH-1, is 2.3 kb. cDNA clones were sequenced on both strands according to published methods (Henikoff, 1987). Sequence databases were searched using BLAST (Altschul et al., 1990) at the National Center for Biotechnology Information and BLOCKS (Henikoff and Henikoff, 1991).

Identification and Characterization of SPH-1 cDNAs

A X. laevis young ovary polyA⁺ cDNA expression library made in the vector Lambda Zap (Stratagene Corp., Burlingame, CA) was screened using mAbH1 (Short et al., 1988). Two independent classes of inserts (700 and 1,900 bp) were isolated from the expression library and subcloned into Bluescript plasmids (Stratagene Corp.). Sequencing of the cDNAs revealed an open reading frame that could encode a 43-kD protein and that the 700-bp insert is identical to an internal region of the 1,900-bp clone. Because the 1,900-bp insert appeared to be lacking the 5' end of the open reading frame (ORF), the 700 bp cDNA was used as a hybridization probe to screen a lambda gt10 library of Xenopus ovary cDNAs to obtain a full-length cDNA (Rebagliati et al., 1985; Maniatis et al., 1982). 15 positive phage were isolated from ~1.2 × 10⁶ phage screened. The largest insert, referred to here as cSPH-1, is 2.3 kb. cDNA clones were sequenced on both strands according to published methods (Henikoff, 1987). Sequence databases were searched using BLAST (Altschul et al., 1990) at the National Center for Biotechnology Information and BLOCKS (Henikoff and Henikoff, 1991).
Expression and Detection of SPH-1 Protein

Epitope tagged SPH-1 protein was expressed in X. laevis oocytes according to previously published methods with minor modifications. The reiterated hexamer epitope tag, MT6 (Roth et al., 1991), was fused to the cSPH-1 sequence encoding either the second methionine (amino acid 25; construct A) or to amino acid 111 (construct B) as follows. The lambda cDNA insert was cloned into a bluescript plasmid (Strategene Corp.) containing the MT6 sequence with the tag ORF preceding, and in the same direction as, the SPH-1 ORF. A construct was then generated by fusing the tag ORF to the cSPH-1 ORF by site directed mutagenesis using a 31 base oligonucleotide sequence encoding either the second methionine (amino acid 25; construct B) or to amino acid 111 (construct A) or to amino acid 111 (construct B) as follows. The lambda eDNA insert was cloned into a bluescript plasmid (Strategene Corp.) containing the MT6 sequence with the tag ORF preceding, and in the same direction as, the SPH-1 ORF. The construct B was made by cloning the original 1,900-bp Lambda Zap cDNA into the MT6 Bluescript plasmid; the 1,900-bp insert encodes the SPH-1 protein from amino acid 111 to the termination codon after amino acid 536. Capped runoff in vitro synthesized transcripts encoding fusion proteins were prepared (Roth and Gall, 1989), and injected into Xenopus oocytes according to methods described by Gurdon et al. (1971). Immunoblotting of pools of 8–20 hand isolated oocyte nuclei 48 h after injection of RNA was done according to Roth et al. (1991). Xenopus lampbrush chromosomes were prepared according to Callan et al. (1987); indirect immunofluorescent staining was done according to Roth and Gall (1987). mAbH10 was isolated by Evan et al. (1985).

Results

Identification and Characterization of a Sphere-specific Monoclonal Antibody

To begin a biochemical characterization of spheres, we developed a method for isolating large numbers of oocyte nuclei (Fig. 2; see Materials and Methods). We immunized mice with X. laevis oocyte nucleoplasm that had been purified away from cytoplasm and nuclear envelopes (Fig. 2, inset). Immunostaining of lampbrush chromosomes with the resulting polyclonal antisera indicated high titers of antibodies against spheres as well as nucleoli, chromosomes, and B snurposomes (data not shown). The spleen cells of one of these mice were fused to a mouse myeloma cell line and the resulting hybridoma cell lines tested for production of antibodies that bind to spheres by indirect immunofluorescent staining of lampbrush chromosome preparations. Of a total of 384 cell lines tested, four produce antibodies that preferentially bind to spheres. After subcloning, four independent hybridoma cell lines were recovered which produce antibodies that bind spheres. While three of the antibodies show some staining of other nuclear structures, one of the antibodies, mAbH1, shows selective binding to spheres with no detectable staining of other nuclear structures (Fig. 3).

Electron microscopic observations of sections through spheres have shown that spheres have morphologically distinct domains including an electron dense core and a cortex that is less dense (Kezer et al., 1980). Confocal microscopy of spheres stained with mAbH1 shows that the antigen is concentrated in the sphere's outer cortex with negligible staining in the inner core (Fig. 4, a and b). Conversely, the inner core is preferentially stained by mAbID7 (Fig. 4, c and d); this antibody binds a conserved epitope on a class of alternative pre-mRNA splicing factors called SR proteins. The staining patterns of mAbH1 and mAbID7 reflect the bipartite structure observed in electron micrographs of spheres (Kezer et al., 1980). Bar, 5 μm.

Figure 3. Indirect immunofluorescent staining of a Xenopus laevis lampbrush chromosome preparation with mAbH1. A and B are phase contrast and fluorescent micrographs, respectively, of the same preparation after staining with the mAbH1. Staining is specific for spheres; the fluorescent images of spheres are somewhat larger than the phase contrast images because of the intensity of fluorescence. Bar, 18 μm.

Figure 4. Confocal microscopy of spheres stained with mAbH1 or mAbID7. A and B show confocal pseudo-phase contrast and fluorescent images, respectively, of a sphere stained with mAbH1; the antigen is localized to the cortex of the sphere. Note that the nucleoli and B snurposomes are not stained. C and D show mAbID7 staining is localized to B snurposomes and the internal core of the sphere. mAbID7 stains B snurposomes both when they are, and when they are not, attached to the surface of spheres. This antibody also stains most of the lateral loops of lampbrush chromosomes and is known to recognize a family of essential splicing factors called SR proteins. The staining patterns of mAbH1 and mAbID7 reflect the bipartite structure observed in electron micrographs of spheres (Kezer et al., 1980). Bar, 5 μm.
cell nuclei, they appear to be much smaller than somatic nuclei; in oocytes spheres and nucleoli are of comparable size.

**Characterization of the mAbH1 Antigen, SPH-1**

To determine the molecular nature of the mAbH1 antigen, oocyte nuclei (germinal vesicles) were extracted in sample buffer and subjected to SDS-PAGE. After transfer to nitrocellulose, the oocyte nuclear proteins were probed with mAbH1 and a single protein of 80 kD was detected (Fig. 6, lane 1). We refer to this 80-kD protein as SPH-1. In addition, mAbH1 binds a band of similar mobility in extracts of Xenopus K2 tissue culture cells (Fig. 6, lane 2).

To isolate a full-length cDNA for this 80-kD protein, two Xenopus ovary cDNA libraries were screened. Initially, we screened ~1 × 10⁶ phage from an expression library with mAbH1 and isolated several overlapping, partial cDNAs of 700 and 1,900 bp. Using the 700-bp expression clone as a probe, we screened a lambda g10 library and identified a 2.3-kb cDNA. This cDNA is referred to as cSPH-1. The nucleotide sequence of cSPH-1 reveals an open reading frame. Nucleic acid and amino acid numbers are shown on the left; amino acids and amino acid numbers are shown in bold type. The putative protein is serine rich but does not appear to have any known functional domains as determined by searching various databases with BLOCKS (Henikoff and Henikoff, 1991) and BLAST (Altschul et al., 1990). There are two short regions of cSPH-1 predicted to be similar to the amino acids 352 and 385. These data sequence are available from EMBL/GenBank/DDBJ under accession number Z23011.

**Figure 7.** Nucleotide sequence of cSPH-1 and predicted amino acid sequence of SPH-1. cSPH-1 is 2.3 kb and contains a 536 amino acid open reading frame. Nucleic acid and amino acid numbers are shown on the left; amino acids and amino acid numbers are shown in bold type. The putative protein is serine rich but does not appear to have any known functional domains as determined by searching various databases with BLOCKS (Henikoff and Henikoff, 1991) and BLAST (Altschul et al., 1990). There are two short regions of RG repeats between amino acids 352 and 385. These data sequence are available from EMBL/GenBank/DDBJ under accession number Z23011.
such constructs were made by fusing the myc tag to the amino terminal region of SPH-1, either to the second methionine (construct A; Fig. 7, bp 301) or to an internal site, deleting the NH2-terminal region of the protein (construct B; Fig. 7, bp 553).

We expressed the fusion proteins by injecting oocytes with capped in vitro synthesized RNA transcripts from either construct A or construct B. 48 h after injection, nuclei were removed from the injected oocytes and tested for expression of the fusion protein. The A fusion protein was unstable and expressed less efficiently than the B fusion protein as detected by immunoblotting (data not shown). Expression from the B construct resulted in production of an 80-83-kD protein that was detectable with both mAbH1 and mAb9E10 (Fig. 8, lanes 1 and 3). Because the predicted size of the B fusion protein is only 58 kD (including 10 kD contributed by the epitope tag), the retarded mobility of this fusion protein is likely to reflect post-translational modification. We assume that the modification occurs on SPH-1 itself because fusion of the same tag to other proteins results in the same mobility shift of only 10 kD when expressed in X. laevis oocyte nuclei. Lampbrush chromosome preparations were made from oocytes that were injected with transcripts encoding epitope-tagged SPH-1 fusion protein B. A is a phase contrast micrograph and B a fluorescent image of a chromosome preparation stained with the anti-tag antibody, mAb9E10. The fusion protein is predominantly localized to spheres. Bar, 20 μm.

Figure 9. Detection of epitope-tagged SPH-1 in a Xenopus laevis lampbrush chromosome preparation. Chromosome preparations were made from oocytes that were injected with transcripts encoding epitope-tagged SPH-1 fusion protein B. A is a phase contrast micrograph and B a fluorescent image of a chromosome preparation stained with the anti-tag antibody, mAb9E10. The fusion protein is predominantly localized to spheres. Bar, 20 μm.

Figure 10. Sequence similarity between SPH-1 and p80-coilin. A search of the protein sequence databases with SPH-1 yields a single protein, p80-coilin, with significant sequence similarity to SPH-1. The COOH-terminal 185 amino acids of SPH-1 and p80-coilin are 54% identical, with an overall similarity of 75%. Amino acid numbers for each protein are shown on the right; p80-coilin amino acid numbers refer to Andrade et al. (1991).

Discussion

The sphere protein we have identified, SPH-1, is the first sphere-specific macromolecule to be characterized. To date, several studies have shown that spheres contain pre-mRNA splicing components (Gall and Callan, 1989; Wu et al., 1991). The antibodies used in this work include mAbY12 which recognizes a conserved epitope on several small nuclear RNA binding proteins (Lerner et al., 1981) and mAbK121 which binds the trimethylguanosine cap structure present on the 5' ends of all the major U-snRNAs except U6 (Krainer, 1988). In addition, both of these antibodies also stain other nuclear structures, including B snurposomes and most lateral loops on lampbrush chromosomes. mAbH1 stains only spheres, indicating that the function of SPH-1 is unique to spheres. The only other known sphere-specific protein is a 120-kD protein identified in the newt Pleurodeles waltl (Lacroix et al., 1985); a cDNA for this protein has yet to be isolated.

Our finding that SPH-1 is similar to p80-coilin is the first piece of evidence that suggests oocyte spheres and somatic cell coiled bodies may be related.
ported by the fact that both spheres and coiled bodies are nuclear structures and by the observation that SPH-1 is present in oocyte and somatic cell nuclei. Furthermore, coiled bodies and spheres are thought to contain snRNPs and snRNAs (Elieieri et al., 1984; Fakan et al., 1984; Wu et al., 1991; Carmo-Fonseca et al., 1992). However, there are apparent differences between spheres and coiled bodies. For example, coiled bodies contain fibrillarin (Andrade et al., 1991) which has been detected in nucleoli (Lischwe et al., 1985) but has not been shown to be in spheres. Similarly, mAbSC35, which stains spheres (Wu et al., 1991), does not stain coiled bodies (Carmo-Fonseca et al., 1991). Hence, the similarity of SPH-1 to p80-coilin is interesting, but further studies are necessary to determine if spheres and coiled bodies are functionally similar.

The identification of SPH-1, together with other studies, more clearly defines the structure of spheres and sheds light on how this organelle is generated. Our working hypothesis is that the formation of spheres is analogous to the formation of nucleoli. Nucleoli arise as a result of rDNA transcription and accumulation of variously assembled pre-ribosomal subunits around the site of transcription (Sheer and Benavente, 1990; Fisher et al., 1991). We propose that spheres arise by a similar mechanism; that is, DNA at the sphere loci is transcribed and a sphere-specific ribonucleoprotein complex (RNP) is formed as a result of specific proteins binding to these nascent transcripts. The accumulation of this RNP gives spheres their distinctive morphology. This model is supported by common features shared by nucleoli and spheres. Each has been mapped to distinct genetic loci on newt lampbrush chromosomes (reviewed in Callan, 1986) and each has an electron dense core and a cortex that is less dense (Kezer et al., 1980; Goessens, 1984). Another feature common to spheres and nucleoli is that they both exist as chromosomally attached and extrachromosomal “free” structures in amphibian oocyte nuclei. Extrachromosomal nucleoli are formed as a result of amplification of rDNA during oogenesis, nucleating the formation of hundreds of free nucleoli (Brown and Dawid, 1968; Gall, 1968). It is possible that sphere DNA is similarly amplified, and that this amplified DNA gives rise to the free spheres. Alternatively, free spheres may arise via “shedding” of accumulated sphere RNP from the chromosomal sphere loci (Callan, 1986). If spheres do arise in the same way as nucleoli, then spheres may be sites of assembly of a distinct RNP. The identification of rDNA as the nucleolar organizer played a fundamental role in understanding the function of nucleoli; we presume that identification of the sphere organizer sequence will be of equal importance for understanding sphere function.

In addition to providing a tool for the direct characterization of spheres, the work presented here provides some useful insight into the possible function of spheres. First, the observation that SPH-1 is present in somatic cells, as well as oocytes, suggests that the function carried out by spheres is not restricted to oocytes, and is therefore likely to be general in nature. Second, preferential staining of the sphere core by mAbID7, which binds a family of pre-mRNA splicing factors called SR proteins (Zahler et al., 1992), suggests that RNA processing may occur in the sphere core. Consistent with this, mAbSC35 (Fu and Maniatis, 1990) which binds the same set of SR proteins as mAbID7 (Neugebauer and Roth, unpublished observations), also stains the core of spheres (Wu et al., 1991). Finally, the observation that the antibody mAbH1 binds the cortex of spheres preferentially suggests that the sphere protein SPH-1 does not directly interact with SR proteins and is therefore unlikely to be involved in pre-mRNA splicing. The binding of the cortex by mAbH1 does suggest, however, that SPH-1 may be a component of an RNP produced by spheres.

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