Large-Scale Isolation of Cajal Bodies from HeLa Cells

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The Cajal body (CB) is a conserved, dynamic nuclear structure that is implicated in various cellular processes, such as the maturation of splicing small nuclear ribonucleoproteins and the assembly of transcription complexes. Here, we report the first procedure for the large-scale purification of CBs from HeLa cell nuclei, resulting in an ~750-fold enrichment of the CB marker protein p80-coilin. Immunofluorescence, immunoblotting, and mass spectrometric analyses showed that the composition of the isolated CBs was similar to that of CBs in situ. The morphology and structure of the isolated CBs, as judged by transmission and scanning electron microscopy analysis, are also similar to those of CBs in situ. This protocol demonstrates the feasibility of isolating intact distinct classes of subnuclear bodies from cultured cells in sufficient yield and purity to allow detailed characterization of their molecular composition, structure, and properties.

INTRODUCTION

An understanding of the structure and organization of the cell nucleus is essential for studying the regulation of cell function and nuclear processes. In both animal and plant cells, nuclear factors involved in events such as DNA replication, transcription, pre-mRNA splicing, and ribosome assembly are organized in spatially distinct nuclear “domains.” These domains include chromosomal territories, nucleoli, interchromatin granule clusters, and various types of nuclear bodies (for review, see Lamond and Earnshaw, 1998; Matera, 1999; Spector, 2001; and Dundr and Misteli, 2001). The mechanisms involved in organizing nuclear body assembly, structure, and movement remain largely unknown. Recent data derived from the expression of fluorescent protein (FP)-tagged fusion proteins in live cells suggest that the interaction of many factors with these nuclear domains is highly dynamic (reviewed by Misteli, 2001). It has been shown that the organization of many nuclear proteins changes during cell differentiation (Antoniou *et al.*, 1993; Santama *et al.*, 1996; Dahm *et al.*, 1998) and can be disrupted in several human diseases, including acute promyelocytic leukemia (Dyck *et al.*, 1994; Koken *et al.*, 1994; Weis *et al.*, 1994) and viral infection (Rebelo *et al.*, 1998).

One of the more intensively studied nuclear domains in recent years is the Cajal body (CB; reviewed by Gall, 2000, 2001). The CB was first described by Ramon-y-Cajal as the “nucleolar-accessory body” in silver nitrate-stained neuronal cells (Cajal, 1903). Later, electron microscopy (EM) studies on neuronal cells showed that this nuclear domain sometimes resembled a ball of coiled threads. The CB can vary in diameter from 0.15 to 1.5 μm or larger. The identification of CBs in the fluorescence microscope was facilitated by the discovery of p80 coilin, a human autoantigen enriched in the CB (Andrade *et al.*, 1991). Antibodies raised against human p80 coilin label CB-like nuclear foci in a wide spectrum of organisms, including plants, suggesting that the CB is a highly conserved structure. Mutagenesis of the gene encoding p80 coilin showed that expression in nuclei of certain mutants can result in not only malformation of CBs but also a general disruption of nuclear organization (Bohmann *et al.*, 1995). The intranuclear distribution of known CB components is affected in cells and tissues from knockout mice lacking the C-terminal 487 amino acids of coilin, suggesting that full-length coilin is important for proper formation and/or maintenance of the CB (Tucker *et al.*, 2001).

Apart from p80 coilin, a growing number of CB components have been identified in recent years. Among them are splicing snRNAs and many small nuclear ribonucleoprotein (snRNP)-specific proteins. Temporal analysis of the localization of newly assembled splicing snRNPs in mammalian cell nuclei showed that they accumulate in CBs before nuclear speckles (Sleeman and Lamond, 1999; Sleeman and Lamond, 1999a; 1999b). Experiments in *Xenopus* oocytes showed that small nucleolar ribonucleoproteins (snoRNPs) also accumulate in CBs before nucleoli (Samarsky *et al.*, 1998; Narayanan *et al.*, 1999; Speckmann *et al.*, 1999). These results suggest a role of the CB in snRNA and/or snoRNA maturation. Some

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Abbreviations used: CB, Cajal body; EM, electron microscopy; FESEM, field emission scanning EM; FP, fluorescent protein; mAb, monoclonal antibody; MS, mass spectrometry; Np, nucleoplasmic fraction; snRNP, small nuclear ribonucleoprotein; TEM, transmission EM studies.

CBs are found to colocalize at specific snRNA gene loci (Smith *et al.*, 1995; Jacobs *et al.*, 1999), suggesting that they may play a regulatory role in snRNA transcription (reviewed by Matera, 1998). In the case of cells containing recombinant arrays of U2 snRNA genes, specific association of CBs was found to depend on U2 snRNA expression from the array (Frey *et al.*, 1999; Frey and Matera, 2001). The recent observations that CBs can physically move within the nucleus (Boudonck *et al.*, 1999; Platani *et al.*, 2000; Snaar *et al.*, 2000) suggest that CBs could be involved in mediating some forms of transport or directed movements of snRNPs in different parts of the nucleus, possibly during their biogenesis and maturation. However, snRNP maturation may be only one of many functions of CBs or one specific example of a more general biological activity that can occur in CBs. For example, CBs also contain proteins involved in other pathways, such as nucleolar functions, tumorigenesis, and cell cycle regulation (Jacobs *et al.*, 1999; Sleeman and Lamond, 1999a; Liu *et al.*, 2000; Ma *et al.*, 2000). It has been suggested that CBs may indeed play a rather general role in *Xenopus* oocytes as centers for the assembly of multiple classes of macromolecular complexes (for review, see Gall, 2000, 2001).

Subcellular fractionation has been an invaluable technique for the development of cell biology, providing numerous insights into the function, structure, and biochemistry of cellular organelles. Over the years, many organelles have been purified, allowing their structures and functions to be studied independently of other cellular components. The advent of high-throughput protein identification by mass spectrometry (MS) has facilitated the large-scale analysis of the protein composition of isolated organelles and multiprotein complexes (reviewed by Andersen and Mann, 2000). Cytoplasmic organelles, which are usually surrounded by membranes and vary in density, are particularly suitable for this approach, thanks to the availability of effective purification procedures. In contrast, it has been difficult to apply this experimental approach to study intranuclear structures, mainly because they are not enveloped by membranes and are therefore hard to purify effectively as intact structures. In the case of mammalian nuclear domains, nucleoli can be effectively isolated because of their high density (Muramatsu *et al.*, 1963). Recently, human nucleoli have been purified from cultured cells and analyzed by MS (Andersen *et al.*, 2002). Nuclear fractions from mouse liver cells that are enriched in interchromatin granule clusters have also been analyzed by MS (Mintz *et al.*, 1999).

Here, we report the first protocol allowing the large-scale purification of CBs from HeLa cells. This establishes the feasibility of isolating distinct classes of subnuclear bodies in sufficient yield and purity to allow detailed characterization of their molecular composition, ultrastructure, and properties.

MATERIALS AND METHODS

Buffers and Solutions

All solutions contained EDTA-free complete protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany), at a concentration of one tablet/50 ml. The compositions of the solutions were as follows: S1 solution, 0.25 M sucrose, 10 mM MgCl₂; S2 solution, 0.35 M sucrose, 0.5 mM MgCl₂; S3 solution, 0.5 M sucrose, 25 mM Tris-HCl, pH 8.5; SP1 buffer, 1 M sucrose, 34.2% Percoll

(Sigma, St. Louis, MO), 22.2 mM Tris-HCl, pH 7.4, 1.11 mM MgCl₂; SP2 buffer, 20% Percoll, 10 mM Tris-HCl, pH 7.4, 1% Triton X100 (BDH, Poole, England), 0.5 mg/ml heparin (Sigma); and HT buffer, 10 mM Tris-HCl, pH 7.4, 1% Triton X100, 0.5 mg/ml heparin.

Antibodies

p80 coilin was detected by mouse monoclonal antibody (mAb) 5P10 (Almeida *et al.*, 1998) or by rabbit antiserum 204/10 (Bohmann *et al.*, 1995). Sm proteins were recognized by mouse mAb Y12 (Lerner *et al.*, 1981). Fibrillarin was detected by mouse mAb 72B9, a kind gift from Professor E.M. Tan of the Scripps Research Institute (Reimer *et al.*, 1987) or by rabbit antiserum, a kind gift from Dr. Francis Fuller-Pace (University of Dundee). Mouse mAb against survival motor neuron (SMN) was purchased from Transduction Laboratories (Lexington, KY). Mouse mAb against SC35 was purchased from Sigma Chemical. Bromouridine 5'-triphosphate was labeled by direct immunofluorescence using an FITC-conjugated mAb originally raised against bromodeoxyuridine (Roche Diagnostics). All fluorochrome-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). All peroxidase-conjugated secondary antibodies were from Pierce (Rockford, IL). All gold-conjugated secondary antibodies were from British BioCell International (Cardiff, UK).

Sonication of HeLa Nuclei

All procedures described below were performed at 4°C unless stated otherwise. HeLa nuclei were purchased from Computer Cell Culture Center (Seneffe, Belgium). After thawing, HeLa nuclei were washed once with S1 solution (1400 × g, 5 min). The nuclei were then resuspended with S1 solution (8 × 10⁷ nuclei/ml) and overlaid on the same volume of S2 solution. After centrifugation (1400 × g, 5 min), the pellet was resuspended at 8 × 10⁷ nuclei/ml in 0.35 M sucrose 0.5 mM MgCl₂. The nuclei were then sonicated with a Misonix 2020 sonicator fitted with a microtip and set at power setting 5. The energy was given in 3 times 6-s pulses, with 6-s intervals between them. To ensure a reproducible delivery of energy, the sonicator was tuned according to manufacturer's instructions, and the nuclei were always sonicated in 3-ml aliquots contained in a 15-ml Corning tube.

Enrichment of CBs

After sonication, 0.42× volume of 2.55 M sucrose was added to 1 volume of the sonicated nuclei, so that the resulting sucrose concentration was 1 M. The nucleoli were pelleted by centrifugation at 3000 × g for 5 min in a GS-6 centrifuge (Beckman, Fullerton, CA), and washed once with S2 solution (1400 × g, 2 min). The supernatant, corresponding to the "nucleoplasmic fraction" (Np), was carefully removed. One volume of the supernatant was mixed thoroughly with 0.8× volume of SP1 buffer. The final volume was measured, and 20% (vol/vol) Triton X100 was added, so that the resulting Triton X100 concentration was 1% (vol/vol). The mixture was loaded into precooled SW41 tubes (Beckman, Palo Alto, CA) and centrifuged in a SW41 rotor (Beckman) at 37,000 rpm for 2 h. After ultracentrifugation, the tubes were carefully unloaded from the top; the bottom 1 ml, containing a loose pellet, was collected and designated as "1P," and the rest of the content was "1S." 1P fractions were pooled and mixed with 0.05× volume of 10 mg/ml heparin (Sigma Chemicals) and 600U/ml DNase1 (Sigma Chemicals). The sample was incubated at room temperature for 30 min, and then mixed with 1× volume of SP2 buffer. The mixture was loaded to precooled SW55 tubes and centrifuged in a SW55 rotor at 45,000 rpm for 1 h. Apart from a loose pellet, a faint white band ~2 cm above the bottom was also visible. The part of the gradient from the top to just above the white band was carefully collected and designated as fraction "2A," the white band was collected as fraction "2B," and the rest of the material, including the pellet, was collected

as fraction "2C." Fractions 2B were pooled and diluted 10 times with HT buffer. The diluted sample was divided into 1.5-ml aliquots and centrifuged at 14,000 rpm in a bench-top microfuge (Eppendorf) for 20 min. The pellets of all aliquots were pooled and recentrifuged so that all material from fraction 2B resulted in one pellet, which was then resuspended in 0.5 ml of S3 solution. The resuspended pellet was centrifuged at 8000 rpm for 5 min in a bench-top microfuge (Eppendorf). The supernatant was carefully removed and designated as fraction 3S, and the pellet was fraction 3P. Fraction 3S, which contained enriched CBs, was diluted 10 times with 25 mM Tris-HCl, pH 8.5, and was pelleted in a microfuge as above.

Immunodetection

To detect the presence of CBs, samples from each of the above fractions were diluted 10 times in TM buffer (10 mM Tris-HCl, pH 7.4, 0.5 mM MgCl₂) and centrifuged in an Eppendorf (Hamburg, Germany) bench-top microfuge (14,000 rpm, 15 min). The pellets were resuspended in <10 µl of TM buffer and spotted onto poly-L-lysine-coated glass microscope slides. The slides were air-dried, rehydrated in PBS, and labeled with various antibodies according to our standard indirect immunofluorescence protocol (Lyon *et al.*, 1997). In some experiments, the preparations were counterstained with Pyronin Y (Sigma Chemicals) after immunolabeling to reveal nucleoli.

For Western analysis, the pellets were resuspended with Novex electrophoresis sample buffer (Invitrogen, Carlsbad, CA), separated in precast gradient polyacrylamide gels (Invitrogen), and blotted onto nitrocellulose membranes according to the manufacturer's instructions. The membranes were blocked in PBS containing 5% (wt/vol) skim milk (Marvel) and 0.1% Tween 20 (BDH) for 1 h at room temperature and immunostained with various antibodies as indicated in the RESULTS section. For Western blotting experiments, in which the amounts of protein loaded per lane were standardized, the protein concentration of each sample was assayed by use of Coomassie Plus Protein Assay Reagent Kit (Pierce), according to manufacturer's instructions and using BSA as standard. The electrochemiluminescence signals were detected with a CCD camera (Fujifilm LAS-1000; Fujifilm, Toyto, Japan) and quantified by use of Aida200 software (Raytest Isotopenmessgeräte GmbH, Straubenhardt, Germany).

Microscopy

Fluorescence microscopy of fixed cells was carried out with a 40× numerical aperture (NA) 1.3, 63× NA 1.4, or 100× NA 1.4 Plan-Apochromat objective. Three-dimensional images and sections were recorded either on an LSM410 confocal microscope (Carl Zeiss; Thornwood, NY) or on a DeltaVision Restoration microscope (Applied Precision, Issaquah, WA) equipped with a three-dimensional motorized stage. Deconvolution of images was carried out with Softworx software (Applied Precision). All images presented here are single optical sections.

For transmission EM (TEM) studies, HeLa cells were pelleted in a microfuge and lightly fixed with 4% paraformaldehyde in PBS for 10 min before they were immunolabeled with anti-coilin (5P10, undiluted hybridoma supernatant, or 204/10, 1:250) and/or anti-SMN (1:5) and 5- and 10-nm gold-conjugated secondary antibodies (1:25). Blocking and antibody dilution buffer was PBS, 0.5% goat serum, 0.1% Tween 20, 1% BSA. Labeled cells were embedded in standard epoxy resin (Durcupan, Sigma) embedding techniques. To analyze isolated CBs, we loaded the fraction containing highly enriched CBs onto poly-L-lysine-coated glass coverslips; it was labeled with anti-coilin and/or anti-SMN antibodies and detected using a combination of fluorescence and gold-conjugated secondary antibodies. Coverslips were examined in the fluorescence microscope, and areas containing a high concentration of labeled CBs were located. Coverslips were then fixed in 80 mM PIPES/KOH, pH 6.8, 1 mM MgCl₂, 1 mM EGTA, 150 mM sucrose, 0.25% glutaraldehyde, and 2% paraformaldehyde; washed in PBS and then in H₂O; postfixed in 1% osmium tetroxide in H₂O for 20 min at room temperature; washed in H₂O; dehydrated in 70% ethanol for 10 min; stained in 1% uranyl acetate in 70% ethanol for 20 min; washed 2 times in 70% ethanol; and further dehydrated through 90, 95, and 100% ethanol and propylene oxide before they were flat-embedded in epoxy resin (Durcupan). Coverslips were removed from the resin by brief immersion in liquid nitrogen. The coverslips could then be snapped off the surface of the resin. Thin sections were cut (Reichert-Jung Ultracut UCT, Leica Microsystem, Nussloch, Germany) and stained with lead citrate before they were examined with a Joel 1200EX transmission electron microscope (Tokyo, Japan).

For field emission scanning EM (FESEM), samples were prepared according to methods described by Goldberg and Allen (1992). Briefly, purified CBs were resuspended in 10 mM Tris-HCl, pH 8.5, and loaded onto poly-L-lysine-coated silicon chips (Agar Scientific Ltd, Stansted, United Kingdom). Unfixed CBs were labeled with anti-coilin antibody and 15 nM gold-conjugated secondary antibodies before they were fixed using SEM fix (80 mM PIPES/KOH, pH 6.8, 1 mM MgCl₂, 1 mM EGTA, 150 mM sucrose, 0.25% glutaraldehyde, 2% paraformaldehyde). Labeled CBs were then dehydrated through a graded ethanol series (70, 90, 95, and 3 times 100%) and then into 100% acetone before they were critical-point dried (Bal-Tec CPD 030, Balzers, Switzerland). Dried specimens were coated with 1.5 nM of chromium and examined in a FESEM (Hitachi S4700, Tokyo, Japan).

RESULTS

Starting Material

The starting material for isolating CBs was nuclei purified from HeLa cells. As the first step in isolating CBs, we used

Figure 1. Effect of magnesium concentration on the degree of disruption of HeLa nuclei by sonication. DIC images of HeLa nuclei sonicated for 18 s in the presence of (A) 0 mM, (B) 0.5 mM, (C) 1 mM, or (D) 5 mM MgCl₂. The sonicated nuclei were immunolabeled with anti-p80 coilin antibody 5P10 to reveal the coilin-containing structures (green). Bars, 5 µm.

Figure 2. Effect of sonication on the morphology of nuclear structures. HeLa nuclei were sonicated for 18 s in the presence of 0.5 mM MgCl_2 and immunolabeled with antibodies raised against (A) p80 coilin, (B) PML, (C) Sm, or (D) fibrillarin. E, F, G, and H are the DIC images of A, B, C, and D, respectively. Bars, 5 μm .

sonication to disrupt nuclei and detach the CBs from other nuclear material while keeping the CBs intact. To optimize this procedure, we compared the use of different salt and buffer conditions during sonication. This showed that for a constant sonication time, magnesium concentration was a crucial factor in the effectiveness of nuclear disruption and CB separation (Figure 1 and other data not shown). In the absence of magnesium (Figure 1A), a brief (18-s) sonication resulted in fragmentation of the nuclei into small particles. Under these conditions, no intact CB labeling could be detected by fluorescence microscopy. At ≥ 0.5 mM magnesium, coilin-positive bodies were detectable after sonication (Figure 1, B–D, arrows). At increasing magnesium concentrations, the nuclei became progressively resistant to physical disruption. However, coilin-positive bodies were detected entangled with clumps of nuclear material at magnesium concentrations 1 mM (Figure 1, C and D). This effect may be a result of changes in the degree of chromatin condensation, a phenomenon sensitive to magnesium concentration *in vitro* (Bojanowski and Ingber, 1998). As judged by a combination of phase contrast and immunofluorescence microscopy, the coilin-containing bodies were best separated from other nuclear material at 0.5 mM magnesium. These bodies appeared to be comparable in size, morphology, and composition to CBs *in situ* (see below).

The effect of sonication on the morphology of other nuclear substructures was also studied. For this analysis, the sonicated nuclei were immobilized on poly-L-lysine-coated glass slides and immunolabeled with antibodies specific for known protein components of nuclear bodies. As discussed above, anti-coilin antibodies labeled discrete foci that were usually not associated with other unlabeled nuclear mate-

rial, as judged by phase contrast microscopy (Figure 2A). Similarly, anti-promyelocytic leukocyte (PML) antibodies labeled foci of similar size and shape but distinct from the coilin-containing bodies (Figure 2B; see also below). Anti-Sm antibodies, specific for splicing snRNPs, also labeled bodies (Figure 2C, arrows), as well as other structures of irregular sizes and shapes (Figure 2C, arrowheads). The Sm-labeled foci were identified as CBs because they were also labeled by the anti-coilin antibody (data not shown), whereas the irregularly shaped structures that were not labeled by the anti-coilin antibody are likely to correspond to clusters of interchromatin granules, or speckles. An anti-fibrillarin antibody labeled nucleoli (Figure 2D, arrowheads) in the sonicated nuclear material, in addition to some foci (Figure 2D, arrows). In summary, sonication of HeLa nuclei at low magnesium concentration appeared to release not only CBs but also other nuclear structures, including PML bodies and nucleoli, free from the bulk of nucleoplasmic material.

Enrichment of CBs

Figure 3 shows a schematic outline of the protocol for isolating CBs from sonicated nuclei. The basic strategy used involved separating CBs from other nuclear material according to (1) density and (2) sensitivity to divalent ion concentration, nuclease treatment, and polyanions. Density separation was carried out with Percoll, a silica sol coated with polyvinylpyrrolidone, which generates a density gradient on ultracentrifugation. The relative speed of gradient formation and the iso-osmolarity of Percoll offer advantages over other gradient materials. We observed when using Percoll-generated nonlinear gradients in the presence of sucrose that

Figure 3. Schematic outline of the protocol for isolation of CBs from HeLa nuclei.

material lighter than a certain cutoff density moved to the top of the tube, whereas denser material moved to the bottom. The cutoff density could be adjusted precisely and reproducibly by changing the concentration of sucrose in the mixture, providing a useful and rapid separation step. Therefore, using carefully selected Percoll-sucrose combinations, it proved possible to separate CBs from other nuclear material. In between each gradient step, the CB-containing fraction was treated to adjust the density such that the major

contaminating material would be removed in the next gradient (see below). Throughout the procedure, both the recovery efficiency and purity of CBs were monitored by a combination of immunocytochemistry and protein blotting to detect known CB components.

In the first step of the procedure, nucleoli were removed from the rest of the nucleoplasmic material by a low-speed centrifugation in 1 M sucrose. Immunolabeling confirmed that most of the CBs were in the Np (Figure 4B), whereas a small number of CBs were selectively associated with the nucleolar periphery (Figure 4C, arrow), as commonly observed *in vivo*. After the removal of nucleoli, the Np was treated with 1% Triton X100 to reduce the density of non-CB particles, whereas a brief increase of magnesium concentration from 0.5 to 0.78 mM selectively increased the density of CBs (data not shown). As a result, a Percoll-sucrose gradient could be designed to separate the CB-containing fraction (fraction 1P, Figure 4E) from other nucleoplasmic material (fraction 1S, Figure 4D). In fraction 1P, the CBs present were mostly entangled with large pieces of chromatin, as revealed by DAPI staining (data not shown). Nucleoli that had not been removed in the first step were also a significant contaminant. To remove chromatin, fraction 1P was treated with DNase and heparin, which is known to increase chromatin accessibility to nucleases (Villeponteau, 1992). At 0.5 mg/ml, heparin dispersed much of the contaminating material into very small particles, whereas the size and morphology of CBs showed little or no change (data not shown). The increased viscosity of the mixture caused by the release of DNA from chromatin was resolved by DNase I treatment. The DNase-heparin-treated fraction 1P was then fractionated by a second Percoll-sucrose gradient, which was designed such that CBs were concentrated in a band (fraction 2B, Figure 4K) in the middle of the gradient, where the resolution is greatest. The majority of CBs were recovered from the gradient in a single thin band, suggesting that they are discrete and relatively homogeneous structures, well separated in density from the majority of other larger nuclear material found in the pellet (fraction 2C, Figure 4M).

Immunofluorescence analysis showed that fraction 2B was enriched with CBs (Figure 4K). Unidentified contaminating material was also revealed by phase contrast microscopy (Figure 4L). Fraction 2B was diluted to lower the concentrations of sucrose and Percoll (see Figure 3 and MATERIALS AND METHODS). The diluted fraction was divided into 1.5-ml aliquots and centrifuged in a microcentrifuge. The pellets were then pooled and recentrifuged, causing the contaminating material to aggregate and allowing its subsequent separation from CBs by a final sucrose cushion (see Figure 3 and MATERIALS AND METHODS). It was also observed that increasing the pH from 7.4 to 8.5 resulted in apparent dissociation of the contaminating material with little or no effect on CBs. The resulting fraction 3S contained a high density of CBs with minimal contaminating material, as judged by phase contrast microscopy (Figure 4Q, insets, arrows).

Analysis of the Enriched CB Fraction

The relative enrichment and yield of CBs after each purification step is summarized in Figure 5. The quantity of CBs in each fraction was estimated on the basis of the relative amount of p80-coilin, as detected by immunoblotting using

Figure 4. Progressive enrichment of coilin-positive bodies during the isolation procedure. Fractions were collected throughout the CB isolation procedure and immunolabeled with anti-coilin antibody 5P10 (green). Nucleoli were revealed by pyronin Y counterstaining (red). Please refer to Figure 3 for fraction nomenclature. (A) Sonicated nuclei, (B) fraction Np, (C) fraction No, (D) fraction 1S, (E) fraction 1P, (K) fraction 2B, (M) fraction 2C, (Q) fraction 3S, and (O) fraction 3P. Insets in Q are close-ups of boxed area. F, G, H, I, J, L, N, and P are the DIC images of A, B, C, D, E, K, M, and O, respectively. Bars for all except Q, 5 μ m; for Q, 25 μ m.

the mouse anti-coilin mAb 5P10. More than 70% of p80-coilin was lost after the first gradient step. Immunofluorescence analysis, however, showed a significant enrichment of CBs in fraction 1P (Figure 4E), whereas fraction 1S (Figure 4D) contained few detectable CBs. The difference between

the immunoblot and immunofluorescence results is most likely explained by the fact that the coilin lost in the first gradient was not assembled into CBs but rather was present in a diffuse nucleoplasmic pool. The existence of a substantial pool of nucleoplasmic p80 coilin has been suggested

Figure 5. Relative enrichment and recovery of p80 coilin during the CB isolation procedure. Selected fractions were collected during the procedure, denatured, and reduced. Then 2.25 μ g of proteins from each fraction was separated by SDS-PAGE and blotted onto a nitrocellulose membrane, which was immunolabeled with anti-p80 coilin antibody 5P10.

previously (Carmo-Fonseca *et al.*, 1993; Bellini and Gall, 1998; Matera, 1998) and is observed in EM studies (Puvion-Dutilleul *et al.*, 1995) and in living cells expressing green FP-coilin (Platani *et al.*, 2000). The present data are therefore consistent with expectations based on the known nuclear distribution of p80 coilin. The isolation procedure thus preferentially enriches the p80 coilin assembled in CBs and thereby provides a method to separate the two pools of coilin. After the final step of the procedure, coilin was enriched by $\sim$ 750-fold relative to the total amount of coilin in the nucleus, with a recovery of $\sim$ 14%. We estimate that the enriched CBs account for $\sim$ 0.02% of the total protein weight of the HeLa nucleus (Figure 5).

The enriched CB fraction was analyzed for the presence of known CB components by both immunofluorescence and protein blotting assays. Proteins from the enriched CB fraction (3S) were separated by SDS-PAGE, transferred to nitrocellulose, and probed with separate antisera specific for the p80 coilin, SMN, fibrillarin, and Sm proteins, respectively. Both p80 coilin and SMN were highly enriched in fraction 3S, which also contained a small subset of fibrillarin and Sm (Figure 6A, lane 9). Figure 6B shows the protein profiles of crude nuclear lysate (lane 1), enriched CBs (fraction 3S, lane 2), and fraction 3P (lane 3). The CB sample includes multiple protein bands that are enriched relative to the control sample that lacks CBs and therefore are likely to include specific CB components (Figure 6B, lane 2, arrows). In addition to the CB components detected by immunoblotting, preliminary analysis of the enriched CB proteins by MS has identified a number of known CB factors, including dyskerin (Heiss *et al.*, 1999), NOP10 (Pogacic *et al.*, 2000), and the human homologue of Nop5/Nop58 (Lyman *et al.*, 1999). The MS analysis also identified additional proteins not previously known to be CB factors, including NHPX, a nucleolar protein known to bind both snoRNAs and U4 snRNA (Watkins *et al.*, 1998; Nottrott *et al.*, 1999). A combination of antibody staining and FP-tagging experiments has confirmed the localization of NHPX to CBs (Leung and Lamond, 2002). A detailed MS analysis of the protein composition of the purified CB fraction is now in progress and will be documented elsewhere.

The enriched CBs were labeled by anti-coilin, anti-SMN, and anti-fibrillarin antibodies but not by anti-PML and anti-SC35 (Figure 7). These data indicate that the isolated CBs have a protein composition similar to that of CBs in intact nuclei. It is worth noting that fraction 3S contained very low levels of PML bodies (Figure 7, J and L, arrows), but their rarity, $<1\%$ of isolated particles, shows that the protocol is specific for isolating CBs rather than other nuclear bodies.

This specificity is further confirmed by the absence of SC35 speckles from the purified CB preparation (Figure 7, M–O).

Ultrastructure of Isolated CBs

Unlike the prominent CBs in primary neuronal cells, CBs in most cultured cell lines, including HeLa cells, less obviously display the "coiled thread" morphology when viewed by EM. Instead, they often appear as electron-dense bodies in the nucleoplasm when analyzed by TEM (Figure 8A). We examined the purified material in fraction 3S by TEM. Almost all the material in this fraction was electron-dense particles similar in size to the CBs found in situ (compare Figure 8, B and C), and these particles were strongly labeled by the anti-coilin antibody (arrows), confirming that they are isolated CBs. The homogeneity of morphology between individual particles and the consistent anti-coilin labeling indicate that the CB preparation is relatively pure. The isolated CBs appeared less regular and round than the native CBs,

Figure 6. (A) Relative amount of p80 coilin, SMN, fibrillarin, and Sm in fractions collected during the CB isolation procedures. From each fraction, 2 μ g of protein was loaded per lane. Lane 1, fraction Np; lane 2, fraction Np, Triton X100 supernatant; lane 3, fraction Np after Triton X100 treatment; lane 4, fraction 1S; lane 5, fraction 1P; lane 6, fraction 1P after DNase and heparin treatment; lane 7, fraction 2A; lane 8, fraction 2B; lane 9, fraction 3S; lane 10, fraction 3P; lane 11, fraction 2C. (B) Protein profile of (lane 1) sonicated nuclei, (lane 2) fraction 3S, (lane 3) fraction 3P. Please refer to Figure 3 for fraction nomenclature.

Figure 7. Composition of isolated CBs. Fraction 3S was double-labeled with anti-coilin antibody 204/10 (red) and one of the following antibodies (green): (A–C) anti-SMN, (D–F) anti-fibrillarin antibody 72B9, (G–I) anti-Sm antibody Y12, (J–L) anti-PML, and (K–N) anti-SC35. Bars, 5 μ m.

Figure 8. TEM analysis of CBs in situ and ex situ. (A–C) HeLa cell or fraction 3S labeled with anti-p80 coilin antibody 5P10 (10-nm gold particles, arrows). (D–F) HeLa cells and fraction 3S double-labeled with anti-p80 coilin antibody 204/10 (5-nm gold particles, arrows) and anti-SMN antibody (10-nm gold particles, arrowheads). (A, D) HeLa nuclei; (B, E) close-up of the CB in A and D, respectively; (C, F) fraction 3S. Bars, 500 nm.

possibly because of either the loss of structural support provided by the surrounding nuclear material or deformation resulting from the isolation protocol. We also examined the location of SMN in the isolated CBs. In the HeLa cells used as starting material, coilin and SMN were colocalized in the same electron-dense bodies (Figure 8, D and E). In isolated CBs, coilin (arrows) and SMN (arrowheads) were also colocalized (Figure 8F).

The isolated CB preparation also provided an opportunity to visualize their surface morphology, which in situ is otherwise masked by chromatin and other surrounding nuclear material. Figure 9 shows the isolated CBs examined with an FESEM. This technique allows examination of the surface morphology of cellular structures, such as nuclear pores (Allen *et al.*, 1997), instead of internal structures viewed in a section. Consistent with the TEM analysis, most of the particles examined (>80%) were strongly labeled by the anti-coilin antibody, visualized here in the secondary images that accompany each FESEM image (Figure 9). Many isolated CBs appeared to contain a rod-like structure (Figure 9H),

and different particles may result from different degrees of coiling of this rod-like motif.

DISCUSSION

We describe here the first large-scale purification method for isolating CBs from cultured mammalian cell nuclei. The procedure involves disrupting HeLa cell nuclei by sonication; treatment with detergent, nuclease, and polyanion; and subsequent density gradient fractionation. It results in the enrichment of particles containing known CB factors that are comparable in size, morphology, and composition to CBs detected in situ. These particles therefore correspond to isolated CBs.

A protocol for the large-scale isolation of CBs must satisfy two basic criteria. First, judging from the fact that a CB occupies only a small fraction of the nuclear volume (the diameter of an average CB is 0.2–0.5 μm , and that of an average mammalian nucleus is 10 μm), we expect the

Figure 9. FESEM of isolated CBs. Fraction 3S was immobilized on a silicon chip, labeled with anti-p80 coilin antibody 5P10 (10-nm gold particles), and examined using a FESEM. (A) Area of 5 CBs. (B–K) Close-up of individual CBs in A. Each FESEM image is accompanied, on the right, by a back-scattered image showing the gold particles of anti-p80 coilin labeling. Bars, (A) 0.2 μm ; (B–K) 0.5 μm .

amount of protein recovered after purification to be $\sim 0.05\%$ of the starting material. The number of nuclei to start with must therefore be large enough to allow for the expected low level of protein recovery. We chose HeLa nuclei as the starting material because of their convenience and ready availability in sufficient quantity for CB isolation. The second criterion for the isolation protocol is that the integrity of the isolated bodies, which are not surrounded by membranes, must be maintained after the nuclei are lysed. It has been reported that nuclear bodies remain morphologically intact after physical disruption or salt extraction of nuclear structure (Brasch *et al.*, 1989; Neves *et al.*, 1999). As a first step, we used sonication to disrupt general nuclear structure and detach the CBs from other material as much as possible, while keeping them intact. Sonication is commonly used for disrupting cells before subcellular fractionation procedures. For example, it is well known that HeLa nucleoli can be

effectively released from intact nuclei after a brief sonication in the presence of sucrose and a low concentration of magnesium (Muramatsu *et al.*, 1963). Steroid-sensitive nuclear bodies from rooster cells could also be released by brief sonication (Brasch *et al.*, 1989).

The conditions used to sonicate HeLa nuclei maintained not only the structure of CBs but also that of nucleoli, PML bodies, and splicing speckles. This indicates that under suitable conditions, many nuclear domains remain intact even after the overall nuclear structure is destroyed. As discussed below, EM analysis demonstrated that the conditions used in the isolation protocol caused little or no change to the morphology of the isolated bodies. These bodies are therefore likely to be distinct structures, maintained by interactions between their components, rather than dependent on essential interactions with an underlying skeleton-like nuclear framework. It may therefore be possible to apply this general approach to the isolation and

purification of a range of distinct subnuclear structures, in addition to the CBs analyzed here.

The use of sequential Percoll-sucrose density gradient fractionation has allowed the isolation of nuclear fractions highly enriched in CBs, as judged by both immunofluorescence and immunoblotting analysis. We estimate an enrichment factor of at least 750-fold for the isolated CBs compared with intact nuclei. This figure is consistent with the anticipated level of enrichment necessary to produce CB preparations 50% pure, based on a calculation of the approximate fraction of the nuclear volume occupied by CBs in intact somatic cell nuclei. It is possible that the enrichment level of CBs is actually 750-fold, because this figure is based on the enrichment of p80 coilin, and more than half of the p80 coilin in nuclei is not assembled into CBs. The protocol described is highly reproducible and can be carried out on a scale large enough to generate sufficient material for biochemical analysis. Importantly, it is shown to effectively separate CBs from the more numerous PML bodies found in the HeLa nuclei, with <1% of the nuclear bodies recovered representing PML bodies.

Analysis of the isolated CBs by both TEM and FESEM showed that they have a internal and surface morphology similar to that seen for somatic cell CBs in intact nuclei. This indicates that the principal features of the CB structure remain stable during the isolation procedure. FESEM analysis showed that the purified CB fraction contained particles of rather homogeneous size and morphology, in agreement with the observations using light microscopy and TEM. Some variation is observed in the degree of compaction of the CBs, possibly reflecting a degree of unfolding or partial disruption during the isolation process. However, the isolated CBs provide an opportunity to view their structure at relatively high resolution and in the absence of other nuclear material that may restrict visualization of their surface morphology when analyzed in situ. The high concentration of CBs in the purified preparations allows many more particles to be examined compared with the low frequency of findings of CBs in EM sections of intact cells. The isolated CBs will therefore facilitate future detailed mapping of the relative localization of different proteins in the CB by immunotEM/FESEM microscopy.

We have commenced with a detailed analysis of both protein and RNA components of CBs using material isolated by the procedure described in this study. Unlike standard immunoprecipitation procedures, in which protein complexes are usually solubilized and then affinity-isolated by use of an antibody that binds to one of the components, our purification procedure was designed to enrich nuclear bodies on the basis of their specific density, morphology, and protein composition. As in any isolation procedure, it is possible that the isolated CB preparations contain contaminating proteins and that a number of CB factors may be lost during the purification procedure. Nonetheless, the ability to analyze large numbers of CB particles and to identify components directly by biochemical and MS analyses offers important advantages for characterizing the composition of CBs as opposed to relying exclusively on indirect immunofluorescence and in situ labeling techniques. In future studies, the CB localization of newly identified proteins will be confirmed by FP-tagging and transient expression, as in our recent effort to identify nucleolar proteins (Andersen *et al.*,

2002). The variation of CB components after different metabolic perturbations will also be investigated as recently reported for nucleoli isolated from cultured cells. For example, the inhibition of transcription resulting from treatment of HeLa cells with actinomycin D was shown to enhance the accumulation of at least 11 proteins, including the CB factor p80 coilin, with nucleoli (Andersen *et al.*, 2002). It will be interesting, therefore, to analyze how the CB proteome may be affected by different drug treatments and other modulators of cell activity, such as stress, which is known to affect the nuclear localization of CB components (Lafarga *et al.*, 1998).

In summary, we report here an effective procedure for the large-scale isolation and purification of nucleoplasmic CBs from mammalian somatic cell nuclei. We anticipate that this protocol will greatly aid future biochemical and ultrastructural characterization of the conserved CB domain and may also help to shed new light on its functional role in vivo. We are attempting to extend the use of the general nuclear fractionation protocol described here to facilitate the purification of a variety of other subnuclear structures. Such biochemical approaches will complement parallel cell biological and genetic approaches being used to study the organization of the cell nucleus and can help to improve our understanding of nuclear function.

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