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Coiled bodies in the nucleolus of breast cancer cells

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SUMMARY

Coiled bodies are a special type of small round nuclear body, composed of coiled fibers and granules, especially prominent in the nucleoplasm of highly active cells (Brasch and Ochs (1992) *Exp. Cell Res*. 202, 211-223). Although no specific function has been assigned to coiled bodies, they contain spliceosome snRNAs and proteins, as well as the nucleolar U3 RNA-associated protein fibrillarin. In the present study, we have used antibodies to the coiled body-specific protein p80-coilin, together with double-label immunofluorescence, confocal microscopy and immuno-electron microscopy, to examine the distribution of coiled bodies in a number of different breast cancer cell lines. By immunofluorescence, all cell lines had prominent coiled bodies in the nucleoplasm and several cell lines appeared to have coiled bodies within the nucleolus itself. Double-label immunofluorescence and confocal laser scanning microscopy confirmed the nucleolar localization of coiled bodies. Besides containing p80-coilin, nucleoplasmic and nucleolar coiled bodies contained fibrillarin and Sm proteins. By conventional and immunoelectron microscopy, nucleolar coiled bodies appeared as discrete structures within the nucleolus in a number of different morphotypes, distinct from the normal nucleolar domains of granular component, dense fibrillar component, and fibrillar centers. While the significance of finding coiled bodies in the nucleolus of certain breast cancer cell lines is at present unknown, this represents the first report of coiled bodies and Sm staining in the nucleolus of mammalian cells.

Key words: coiled body, nucleolus, breast cancer

INTRODUCTION

From the electron microscopic examination of pathological specimens, Bouteille et al. (1967) originally described five different types of nuclear bodies that were related to cellular hyperactivity due to physiological, hormonal, drug-induced, viral or malignant conditions (reviewed by Brasch and Ochs, 1992). Many of these nuclear bodies occurred in proximity to the nucleolus and some were observed to 'bud' from the nucleolar surface (Dupuy-Coin and Bouteille, 1972). On the basis of their nuclease and protease sensitivity, they were thought to be composed of complexes of RNA and protein. Nuclear bodies have also been studied in a number of model systems for increased cellular activity, including Con A stimulation of isolated mouse peripheral blood lymphocytes (Chaly et al., 1983a,b), estrogen stimulation of rat uterus (Clark et al., 1978; Padykula and Clark, 1981), and estradiol-induced vitellogenesis in hepatocytes of rooster liver (Brasch and Peters, 1985; Brasch et al., 1989). In all of these model systems, the numbers and complexity of nuclear bodies increased with stimulation of cellular activity, implying a physiological role for nuclear bodies in unidentified nuclear events.

The best-studied nuclear body to date is the coiled body (Raska et al., 1990b; Brasch and Ochs, 1992; Spector, 1993; Lamond and Carmo-Fonseca, 1993). This is due in part to the recent availability of specific antibody probes to the coiled body-specific protein p80-coilin (Raska et al., 1990a, 1991; Andrade et al., 1991, 1993). Coiled bodies were first seen by light microscopy in cell bodies of neurons by the Spanish cytologist Ramon y Cajal in 1903 (reviewed by Lafarga and Hervas, 1983), who referred to them as 'accessory bodies' to denote their close association with nucleoli when visualized by silver impregnation. Coiled bodies were first named and studied in detail in the pioneering electron microscopic studies of Monneron and Bernhard (1969), who described them as round bodies 0.5-1.0 μm in diameter composed of tightly packed coiled fibers. Staining by Bernhard's EDTA-regressive method (Bernhard, 1969) indicated their ribonucleoprotein nature. Coiled bodies have been reported in both plant (Lafontaine, 1965; Moreno Diaz de la Espina et al., 1982a,b; Williams et al., 1983; Chamberland and Lafontaine, 1993) and animal cells (Raska et al., 1990a, 1991; Andrade et al., 1991, 1993), and along with simple nuclear bodies they are the major type of nuclear body found in cultured cells (Raska et al., 1990b). Despite their content of RNA and protein, coiled bodies do not incorporate uridine (Fakan and Bernhard, 1971; Fakan et al., 1976; Moreno Diaz de la Espina et al., 1982a) and they lack any detectable DNA (Monneron and Bernhard, 1969; Raska et al., 1991).

Other than marker protein p80-coilin for which a partial cDNA sequence is known (Andrade et al., 1991), coiled bodies have been reported to contain the U3 snRNP-associ-

ated nucleolar protein fibrillarin, the nucleolus organizer region (NOR) silver staining protein, DNA topoisomerase I, the U1,U2,U4,U6 snRNP-associated Sm proteins, and the U2 snRNP auxiliary splicing factor U2AF (Fakan et al., 1984; Eliceiri and Ryerse, 1984; Raska et al., 1990a, 1991; Zhang et al., 1992). Coiled bodies do not have detectable amounts of nucleolar proteins B23, nucleolin, RNA polymerase I, 5 S rRNP, splicing factor SC35, hnRNP protein L, or interchromatin granule protein 3C5 (Raska et al., 1991). Using in situ hybridization with oligonucleotide antisense probes, coiled bodies have been reported to contain U2, U4, U5, U6 and U12 snRNAs, but no detectable RNAs for U1, U3, 7SK, 5 S RNA or rRNA (Carmo-Fonseca et al., 1991a,b, 1993; Matera and Ward, 1993). Therefore, coiled bodies are somewhat enigmatic structures, since they contain protein and RNA molecules involved in the metabolism of both pre-rRNA and pre-mRNA (reviewed by Lamond and Carmo-Fonseca, 1993). Coiled bodies may be analogous to the snRNP-containing sphere organelles or snurposomes of amphibian oocytes that are associated with active sites of transcription on lampbrush chromosomes, and as free bodies in the nucleoplasm may be sites for assembly and/or storage of snRNP complexes (Gall and Callan, 1989; Gall, 1991; Wu et al., 1991; Tuma et al., 1993).

Coiled bodies vary in number and size throughout the cell cycle and in different cell types (Andrade et al., 1991, 1993; Raska et al., 1991), with fewer coiled bodies in contact-inhibited cells of defined passage, more in immortal cells, and the highest numbers in transformed cells (Spector et al., 1992). In general, coiled bodies are not detectable during mitosis even though the total amount of p80-coilin remains constant throughout all stages of the cell cycle (Andrade et al., 1993), and presumably this is due to M-phase phosphorylation of coilin and subsequent disassembly of coiled bodies (Carmo-Fonseca et al., 1993). Coiled bodies are sensitive to the proliferative state of the cell as well, since their expression can be 'down regulated' by serum starvation of 3T3 cells and 'up regulated' by refeeding or by the addition of the thyroid hormone TSH (thyrotropin) to hormone-depleted rat thyroid FRTL-5 cells (Andrade et al., 1993). In the only functional studies to date, inhibition or alteration of transcription by actinomycin D, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), α-amanitin, or heat-shock displaced snRNPs from coiled bodies and resulted in the association of p80-coilin with the nucleolus (Raska et al., 1990a; Carmo-Fonseca et al., 1992). All of these studies serve to demonstrate the dynamic nature of coiled body expression and its sensitivity to a changing cell metabolism.

In a previous paper (Brasch and Ochs, 1992), we reported an increase in nuclear coiled bodies in liver hepatocytes after injection of β-estradiol into young male roosters. In order to better examine hormone-induced expression of coiled bodies in an in vitro system, we studied the distribution of coiled bodies in a number of estrogen-sensitive human breast cancer cell lines. In this paper, we report finding coiled bodies in nucleoli of some, but not all, cell lines of breast cancer origin. Like nucleoplasmic coiled bodies, nucleolar coiled bodies contained p80-coilin, fibrillarin and Sm proteins. These results are discussed relative to coiled body function, breast cancer and the possible nucleolar origin of coiled bodies.

MATERIALS AND METHODS

Cells and cell culture

HEp-2, MOLT-4, HBL-100, T47D and MCF-7 cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD). MCF-7 variants MCF-7/AZ and MCF-7/6 were a gift from Dr James Freeman, Lucille Markey Cancer Center, University of Kentucky College of Medicine, Lexington, Kentucky. HEp-2 cells were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 10 μg/ml gentamicin sulfate. MOLT-4 cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 10 μg/ml gentamicin sulfate. Human breast cell lines HBL-100, T47D, MCF-7, MCF-7/AZ and MCF-7/6 were grown in RPMI-1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 μg/ml gentamicin sulfate, and 0.25 i.u./ml insulin (Sigma, St Louis, MO).

Human breast cell line HBL-100 originated from a primary culture of breast milk from a lactating female (Gaffney, 1982), whereas human breast cancer cell lines T47D (Keydar et al., 1979) and MCF-7 (Soule et al., 1973) were established from pleural effusions of patients with breast carcinoma. MCF-7/AZ and MCF-7/6 cell lines were derived from parental MCF-7 cells and a characterization of these cells has been reported (Bracke et al., 1991; Coopman et al., 1991).

Antibodies

Antibodies were obtained from the serum bank of the W. M. Keck Autoimmune Disease Center and were previously characterized as follows: rabbit R288 antibody to p80-coilin (Andrade et al., 1993), human autoantibody to fibrillarin (Ochs et al., 1985; Lischwe et al., 1985), human autoantibody to RNA polymerase I (Reimer et al., 1987b), human autoantibody NOR-90 to ribosomal RNA transcription factor UBF (Chan et al., 1991), and mouse monoclonal antibody 72B9 to fibrillarin (Reimer et al., 1987a). The human autoantibody to Sm proteins used in this study was characterized by western blotting (see Fig. 8B, below). All antibodies were used at a dilution of 1/100.

Immunofluorescence and confocal laser scanning microscopy

In preparation for indirect immunofluorescence and confocal laser scanning microscopy, cells were grown on glass coverslips, rinsed briefly with 10 mM phosphate-buffered saline (PBS), and then fixed for 30 minutes in 3% paraformaldehyde/PBS followed by 100% acetone for 1 minute at −20°C. For double-labeling, combinations of human autoantibodies were used with different rabbit polyclonal or mouse monoclonal antibodies followed by affinity-purified second antibodies consisting of FITC-conjugated goat anti-human or anti-rabbit IgG and RITC-conjugated goat anti-mouse or anti-human IgG, all obtained from Caltag (South San Francisco, CA). Coverslips were mounted on glass slides with Vectashield mounting medium (Vector Labs, Burlingame, CA) to minimize quenching of the fluorescence signal.

For confocal microscopy, 0.4 μm optical sections of double-labeled cells were recorded with a Bio-Rad MRC-600 confocal laser scanning microscope equipped with an argon/krypton laser and a Zeiss IM-35 inverted microscope with a ×63/1.4 NA oil immersion objective lens. Confocal images were collected simultaneously from the fluorescein and rhodamine channels and merged using a Bio-Rad CoMos software program. To separate both channels, a dichroic beamsplitter was used. Final images were obtained as slides taken from a film recorder with Kodak HC-100 film. Photographic prints were then made from the color transparancies.

Electron microscopy and immunoelectron microscopy

For electron microscopy and immunoelectron microscopy, cells were grown as monolayers in Lux Permanox dishes (Electron Microscopy

Sciences, Fort Washington, PA). After 2 days in culture, monolayer cells were rinsed with PBS, fixed 1 hour at room temperature in 2.5% glutaraldehyde buffered with 0.1 M cacodylate (pH 7.2), rinsed in cacodylate buffer, postfixed for 1 hour in 2% OsO_4 buffered with cacodylate, dehydrated in a graded ethanol series, and embedded in Polybed 812 (Polysciences, Warrington, PA). Thin sections were stained with uranyl acetate and lead citrate.

For peroxidase pre-embedding immunoelectron microscopy, cells were fixed for 30 minutes at room temperature (RT) with 3% paraformaldehyde buffered with PBS, rinsed with PBS, and then permeabilized at −20°C in 100% acetone for 1 minute, as for immunofluorescence. Following a PBS rinse, cells were blocked for 30 minutes at RT with 1% normal goat serum in PBS (NGS/PBS), incubated overnight at 4°C with antibody diluted 1/100 in 1% NGS/PBS, rinsed 3× 10 minutes with PBS, blocked for 30 minutes with 1% NGS/PBS, and then incubated for 1 hour at 37°C with affinity-purified goat anti-human or anti-rabbit IgG coupled to peroxidase (Cappel, Durham, NC) diluted 1/100 in 1% NGS/PBS. Cells were then rinsed 3× 10 minutes in PBS, fixed for 30 minutes at RT with 1% glutaraldehyde buffered with PBS, rinsed in PBS, rinsed in 50 mM Tris-HCl (pH 7.6), and then incubated for 5 minutes at RT in 1 mg/ml diaminobenzidine tetrahydrochloride (Polysciences)/0.03% H_2O_2 in Tris buffer, rinsed with Tris buffer, rinsed with distilled water, and then stained for 30 minutes with 2% OsO_4 in distilled water, rinsed in distilled water, dehydrated with ethanol, and then embedded in Polybed 812. Embedded cells were thin-sectioned as monolayers, and then examined unstained in the electron microscope.

For Nanogold pre-embedding immunoelectron microscopy, monolayer cells were fixed, blocked and incubated overnight in primary antibody as described above for peroxidase immunoelectron microscopy. Following three 10-minute rinses in PBS and blocking for 30 minutes in NGS/PBS, cells were incubated for 1 hour at RT with shaking in 1.4 nm Nanogold (Nanoprobes, Stony Brook, NY) covalently linked to anti-human or anti-rabbit Fab′ diluted 1/100 in NGS/PBS, rinsed in PBS, fixed for 30 minutes in 1% glutaraldehyde/PBS, rinsed in PBS, rinsed well in distilled water, silver-enhanced for 4 minutes in the dark at RT with HQ Silver (Nanoprobes), rinsed with water, osmicated for 30 minutes with 1% OsO_4 in water, rinsed with water, dehydrated with ethanol, and embedded in Polybed 812. Thin sections were examined unstained. Gold particle labeling was quantitated by planametric analysis of EM negatives of thin sections at a magnification of ×10,000 and expressed as number of gold particles/μm^2.

Immunoblotting

Gel electrophoresis and western blotting were done according to the procedure of Chan and Pollard (1992). A 25 μg sample of protein from each whole cell extract was mixed with electrophoresis sample buffer (63 mM Tris-HCl, 10% glycerol, 2.3% SDS, 2% beta-mercaptoethanol, 0.005% bromphenol blue, pH 6.8) and heated at 95°C for 5 minutes prior to separation on a 10% SDS-resolving gel with a 4% stacking gel as described by Laemmli (1970). The gel was calibrated with prestained molecular mass markers (Bio-Rad, Richmond, CA) and protein separation verified by staining with 0.1% Coomassie Blue. For immunoblotting, proteins were electrophoretically transferred to nitrocellulose membranes (S & S, Keene, NH) using 50 V for 2 hours at 4°C as described by Towbin et al. (1979). The transfer efficiency was confirmed by Ponceau S staining. Prior to application of antibody, the nitrocellulose membranes were cut into strips and saturated with 5% nonfat dry milk in PBS (M-PBS) for 1 hour at room temperature. Nitrocellulose strips were then incubated for 45 minutes at room temperature with rabbit R288 polyclonal antibody to p80-coilin or human autoimmune serum to Sm proteins diluted 1/100 in 5% M-PBS. Strips were then washed 3× 30 minutes with PBS-0.2% Tween-20 to remove unbound antibody. Detection of immunoreactive bands was done by incubation with ^{125}I-Protein A (ICN, Costa Mesa, CA) at a specific activity of 0.1 μCi/ml in 5% M-PBS for 45 minutes at room temperature. Strips were washed for 1 hour in PBS-Tween-20 with frequent buffer changes, dried and exposed to Kodak XAR-5 film overnight.

RESULTS

Nuclear bodies containing p80-coilin and Sm proteins are localized within nucleoli of human T47D breast cancer cells

In examining various types of hormone-responsive cells for the presence and distribution of coiled bodies, we initially tested various breast cancer cell lines that were known to have receptors for estradiol. Fig. 1 illustrates our results with the human breast cancer cell line T47D, originally derived from the pleural effusion of a female patient with breast carcinoma (Keydar et al., 1979). Prominent p80-coilin-containing nuclear bodies were detected in the nucleoplasm (arrowheads in Fig. 1A-C) of some, but not all, T47D cells. Smaller and more numerous coilin-containing nuclear bodies were also observed in some of the phase-dense nucleoli (arrows in Fig. 1A-C). Combination phase-contrast and immunofluorescence (Fig. 1B) confirmed the apparent nucleolar localization of these nuclear bodies. Not only were the nucleolar bodies smaller than the nucleoplasmic bodies, their fluorescence intensity was also correspondingly less than that for bodies in the nucleoplasm. Double-label immunofluorescence for the detection of coilin (Fig. 1C) and Sm (Fig. 1D) indicated that both nucleoplasmic (arrowhead in Fig. 1D) and nucleolar bodies (arrows in Fig. 1D) contained Sm proteins as well. Again, the staining was weaker in the nucleolar bodies compared to nucleoplasmic bodies. In addition to staining nucleolar and nucleoplasmic bodies, the classical nucleoplasmic ‘speckled’ Sm pattern of staining was also observed. Since human autoantibodies may have multiple specificities, cells were also stained with monoclonal anti-Sm antibody Y12 in single-labeling experiments (data not shown). Y12 labeling was identical to the human autoantibody, with labeling of nucleolar and nucleoplasmic coiled bodies and nucleoplasmic speckles.

Since coilin-containing nucleolar bodies were round and within the size range for fibrillar centers, we performed double-label immunofluorescence for coilin (Fig. 1F,G) and the RNA polymerase I transcription factor NOR-90/UBF (Fig. 1H) which has been localized to nucleolar fibrillar centers and the dense fibrillar component (Rodrigo et al., 1992; Rendon et al., 1992; Roussel et al., 1993). A comparison of the nucleolar staining patterns in Fig. 1G and H (see arrows) indicated no colocalization between coilin and NOR-90/UBF in nucleolar bodies or elsewhere in the nucleus.

By whole cell immunofluorescence microscopy as shown in Fig. 1, it looked as if nuclear bodies containing p80-coilin and Sm proteins were present within nucleoli of some T47D cells. Taking a more critical point of view, these nucleolar bodies could be located on the peripheral top or bottom edge of the nucleolus, thus appearing to be contained within the nucleolus proper. To definitively investigate this point, we performed optical sectioning at 0.4 μm increments with the confocal laser scanning microscope, along with colocalization for coilin/fibrillarin (Fig. 2A), coilin/NOR-90 (Fig. 2B), and coilin/Sm (Fig. 2C-E). Small 0.5-1.0 μm nuclear bodies containing p80-coilin and fibrillarin were located totally within the nucleolus (single

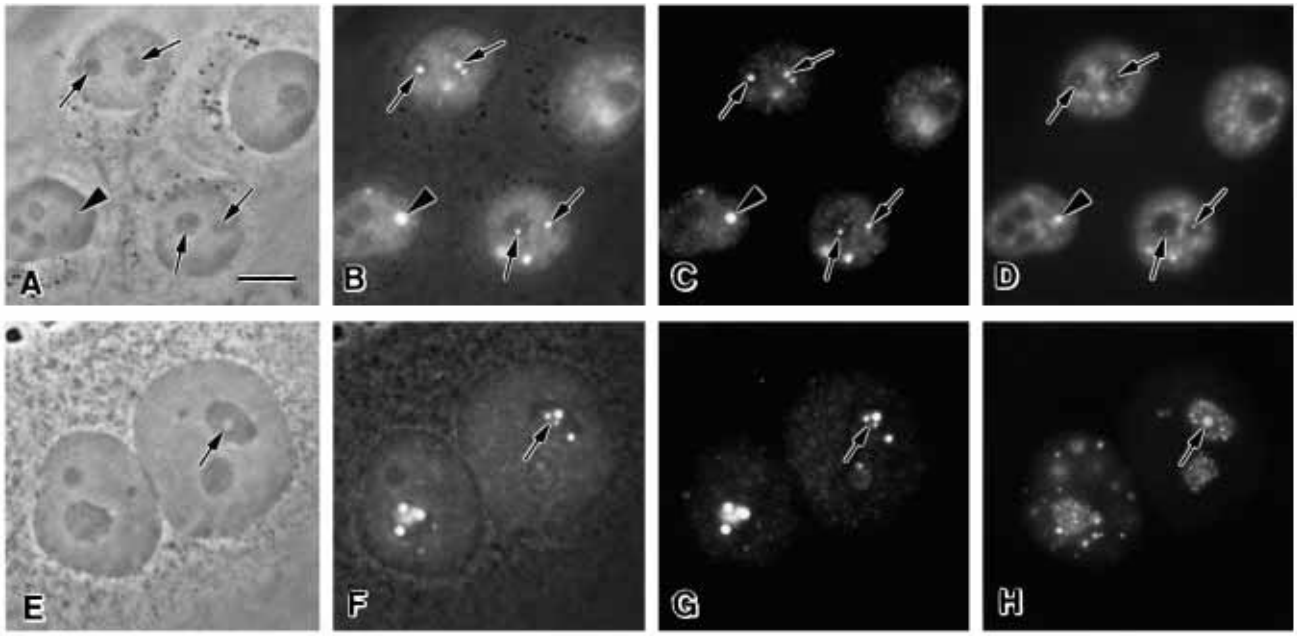

Fig. 1. Phase-contrast microscopy (A,E), combination phase-contrast and immunofluorescence microscopy (B,F), and double-label immunofluorescence (C,D,G,H) localization of p80-coilin (B,C,F,G) compared to the spliceosomal Sm proteins (D) and the ribosomal DNA transcription factor NOR-90/UBF (H) in the human breast cancer cell line T47D. Coilin staining was confined to small round bodies within phase-dense nucleoli (arrows in A-C) and to larger bodies located in the nucleoplasm (arrowheads in A-C). Sm staining, although weaker in intensity, identified these same nuclear bodies (arrows and arrowhead in D) as well as a speckled staining pattern throughout the nucleoplasm excluding nucleoli. In (E-H) coilin was localized in nucleolar bodies (F,G) surrounding a phase-light region of the nucleolus (arrow in E), which stained for NOR-90/UBF (arrow in H). Bar, 10 μm.

arrowheads in Fig. 2A) and on its periphery (double arrowheads in Fig. 2A). Larger bodies, up to 2 μm in diameter, were found free in the nucleoplasm (arrow in Fig. 2A). Using antibodies to NOR-90/UBF as another marker for the nucleolus in double labeling (Fig. 2B), nucleolar bodies containing p80-coilin did not appear to have significant colocalization with NOR-90/UBF and hence appeared more green than yellow in the merged red and green images. As with the double-labeling in Fig. 1C,D, Sm proteins were detected in both nucleolar (arrowheads in Fig. 2C-E) and nucleoplasmic (arrow in Fig. 2C-E) coilin-containing bodies. Not only did confocal microscopy prove that nucleolar bodies containing p80-coilin did exist, but the degree of colocalization with other antigens could be assessed in merged color images of red and green by the amount of yellow staining.

Nuclear bodies are present in nucleoli of other human breast cancer cell lines

Since this is the first report of nuclear bodies containing p80-coilin and Sm staining occurring within the nucleolus of a particular cell line, we tested a number of other human breast cell lines for nucleolar bodies as well. Fig. 3 is a depiction of our results with normal breast cell line HBL-100 (Fig. 3A-C), breast cancer cell line MCF-7 (Fig. 3D-F), and MCF-7-derived breast cancer cell lines MCF-7/AZ (Fig. 3G-I) and MCF-7/6 (Fig. 3J-L). Cell lines HBL-100 and MCF-7 both had coilin-containing bodies in the nucleoplasm (single arrowheads), some of which appeared to be in contact with the nucleolar periphery (double arrowheads), but no bodies were observed within nucleoli. In MCF-7-derived cell lines MCF-7/AZ and MCF-7/6 nuclear bodies containing p80-coilin were present in the nucleoplasm (single arrowheads), on the periphery of nucleoli (double arrowheads), and within nucleoli (arrows). Compared to cell lines HBL-100 and MCF-7, MCF-7/AZ and MCF-7/6 cells demonstrated additional fine-speckled nucleoplasmic staining with antibodies to p80-coilin. The significance of this staining is unknown.

Nucleoli of breast cancer cells contain coiled bodies

To determine the nature of the nuclear bodies occurring in nucleoli of breast cancer cells, we processed different cell lines for conventional electron microscopy (Fig. 4). Breast cancer cell line T47D was composed of cells with multiple nucleoli, some of which contained coiled bodies of various sizes and morphology (Fig. 4A-D). Nucleolar coiled bodies were composed of combinations of coiled fibers (Fig. 4B) or granules (Fig. 4D), and sometimes appeared continuous with the dense fibrillar component of the nucleolus (arrowhead in Fig. 4C). More typically, however, coiled bodies appeared totally separate from the normal nucleolar constituents of fibrillar centers, dense fibrillar component, and the granular component. Of the hundreds of thin sections examined, we never observed invagination of the nucleolus around coiled bodies. Instead, coiled bodies were always completely contained within the nucleolus, leading to the conclusion that they formed from within the nucleolus rather than being engulfed by it.

MCF-7 cells had no coilin-containing nucleolar bodies by immunofluorescence microscopy (Fig. 3D-F), and they also had no nucleolar coiled bodies by conventional electron

microscopy (Fig. 4E). MCF-7/AZ cells, on the other hand, did have nucleolar coiled bodies (Fig. 4F), which looked similar in appearance to nucleoplasmic coiled bodies (inset to Fig. 4F). Therefore, those breast cancer cell lines that had nucleolar bodies containing p80-coilin by immunofluorescence microscopy also had nucleolar coiled bodies by electron microscopy.

Nucleolar coiled bodies contain lower amounts of coilin, Sm and fibrillarin compared to nucleoplasmic coiled bodies

Conventional electron microscopy revealed that classical-looking coiled bodies were present within nucleoli of breast cancer cell lines T47D, MCF-7/AZ and MCF-7/6. To definitively localize p80-coilin-containing bodies within the

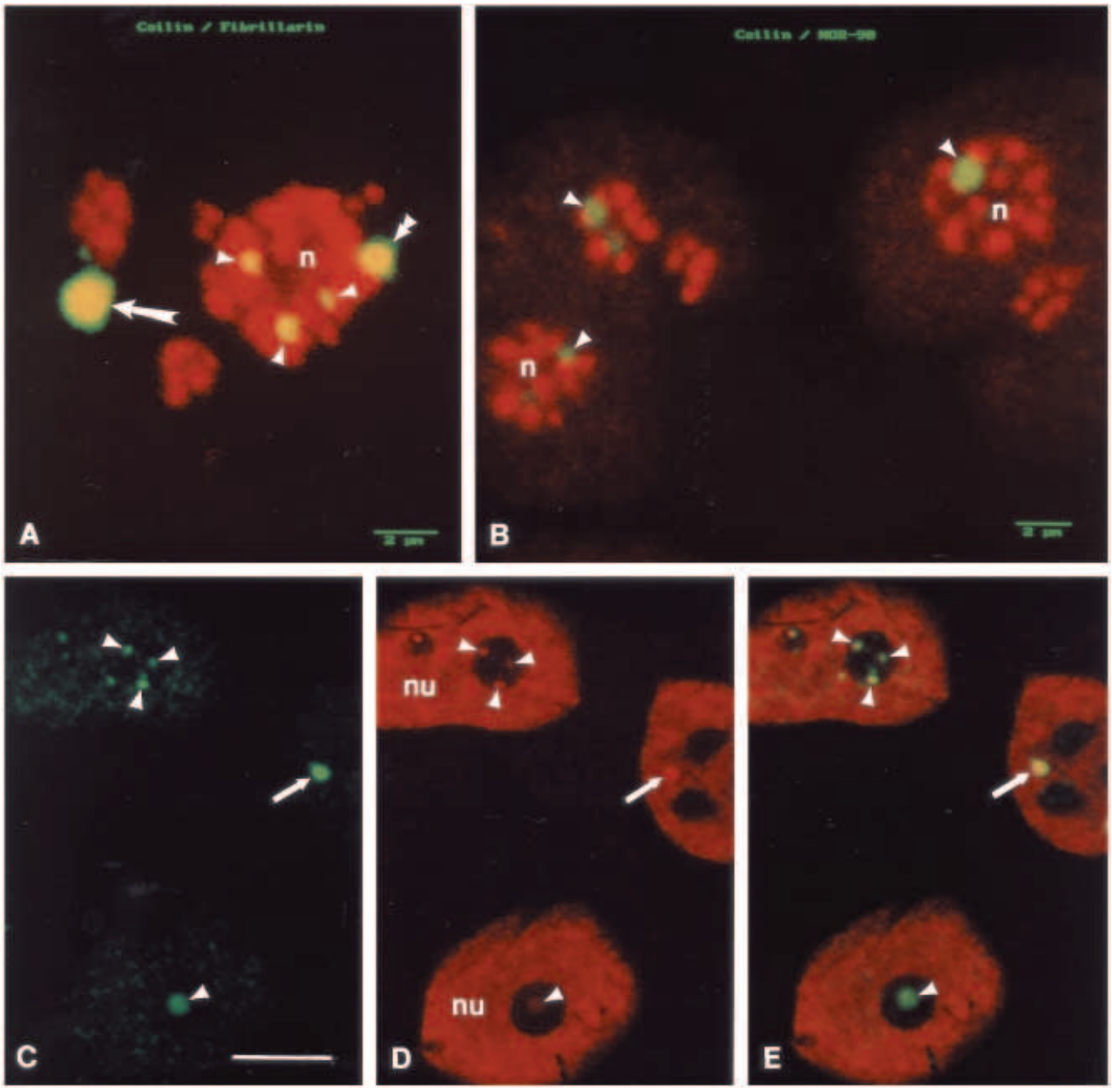

Fig. 2. Optical sections from confocal laser scanning microscopy showing the nucleolar localization of nuclear bodies, which stain for p80-coilin in T47D cells. (A) Optical sectioned and merged image of coilin (green) and fibrillarin (red). Nuclear bodies (single arrowheads) appear within the nucleolus (n), on its periphery (double arrowheads), and in the nucleoplasm (arrow). Yellow staining indicates colocalization by superimposition of red and green staining patterns. (B) Merged image of staining for NOR-90 (red) and coilin (green). The green staining pattern of nuclear bodies (arrowheads) in the nucleolus (n) indicates a lack of significant colocalization. (C-E) Separated (C,D) and merged image (E) of staining for coilin (green staining in C) and Sm (red staining in D). Coilin was localized to bodies within the nucleolus (arrowheads in C) and to a single body in the nucleoplasm (arrow in C). Nuclear bodies were also weakly stained for Sm (arrowheads and arrow in D). The 'yellower' color of the nucleoplasmic body in (E) (arrow) compared to the bodies found in the nucleolus (arrowheads) is a result of the greater concentration of Sm. nu, nucleus. Bars: (A,B), 2 μm; (C-E), 10 μm.

nucleolus, pre-embedding immunoperoxidase electron microscopy was performed as well (Fig. 5). As a positive control, coilin was first localized in HEp-2 cells in small nucleoplasmic bodies (arrowheads in Fig. 5A). In T47D cells, coilin was localized to much larger bodies in the nucleoplasm (arrowhead in Fig. 5B) and within nucleoli (arrows in Fig. 5B). Sm staining in T47D cells was concentrated strongest in nucleoplasmic bodies (arrowhead in Fig. 5C), weaker in nucleoplasmic patches throughout the nucleoplasm (double arrowheads in Fig. 5C), and weakest staining in nucleolar bodies (arrows in Fig. 5D). In MCF-7/AZ cells, coilin was localized to large nucleoplasmic bodies (arrowhead in Fig. 5E), to nucleolar bodies (arrow in Fig. 5F), and to small patches of staining distributed throughout the nucleoplasm (arrowheads in Fig. 5F).

Even though immunoperoxidase electron microscopy allowed us to conclude that coilin and Sm-containing bodies were present within nucleoli, this technique would not allow us to identify these structures unambiguously as coiled bodies, nor would it allow for any measure of quantitation. In order to address these

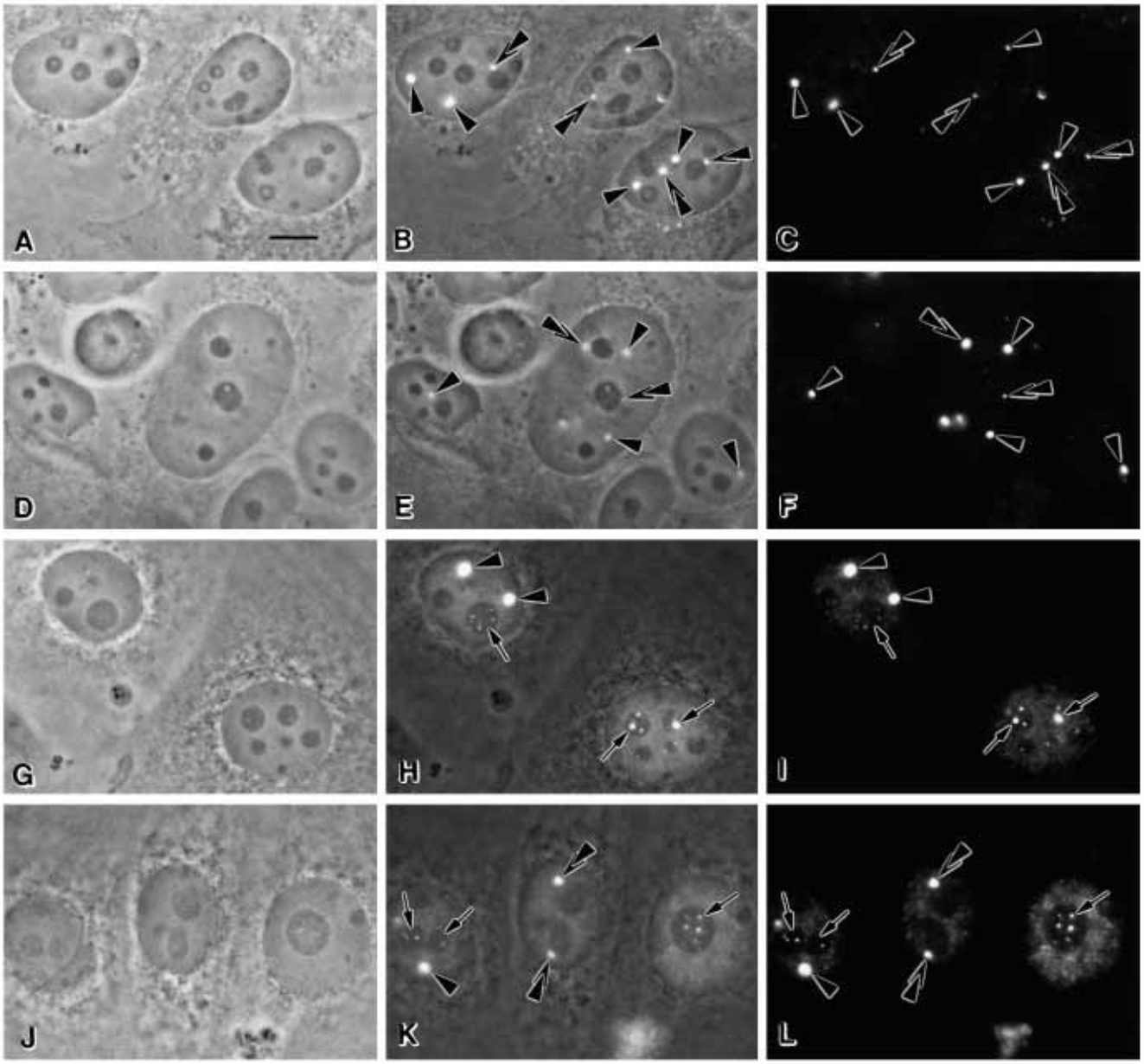

Fig. 3. Localization of coilin in other human breast cell lines. Phase-contrast (A,D,G,J), combination phase-contrast and immunofluorescence (B,E,H,K), and immunofluorescence microscopy (C,F,I,L) of coilin localization in normal breast cell line HBL-100 (A-C), in the breast cancer cell line MCF-7 (D-F), and in MCF-7-derived breast cancer cell lines MCF-7/AZ (G-I) and MCF-7/6 (J-L). Single arrowheads indicate nuclear bodies in the nucleoplasm, double arrowheads indicate nuclear bodies on the periphery of nucleoli, and arrows denote nucleoli that contain nuclear bodies within them. Normal breast cell line HBL-100 (A-C) and parental breast cancer cell line MCF-7 (D-F) do not contain nuclear bodies in their nucleoli, whereas derivative cell lines MCF-7/AZ (G-I) and MCF-7/6 (J-L) do. Bar, 10 μm.

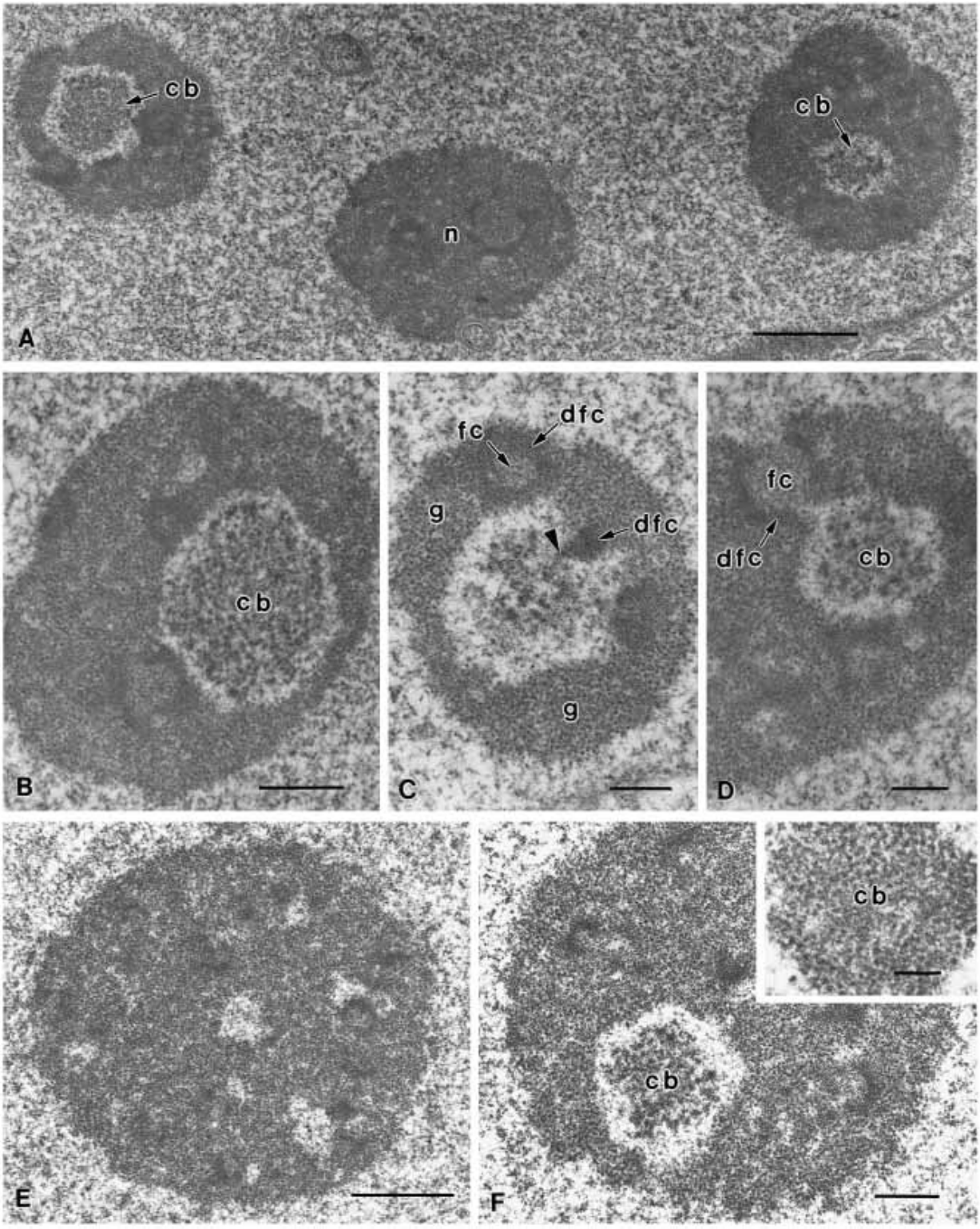

Fig. 4. Electron microscopy of nucleoli from breast cancer cell lines T47D (A-D), MCF-7 (E) and MCF-7/AZ (F). Breast cancer cells have multiple nucleoli (n), some of which contain coiled bodies (cb) in various sizes and appearances (A-D). Some nucleolar coiled bodies appear to be composed of coiled fibrils (B), while others have coarser fibers (C) and structures that look more like granules (D). The coiled body in C appears to be continuous with the dense fibrillar component (dfc) of the nucleolus (arrowhead). A small fibrillar center (fc) surrounded by the dfc is also illustrated. The remainder of the mass of the nucleolus is composed of the preribosome-containing granular component (g). The coiled body in (D) appears to be continuous with a fibrillar center. Nucleoli of the parental cell line MCF-7 (E) contained no coiled bodies by electron microscopy, whereas nucleoli of the derivative cell line MCF-7/AZ (F) contained coiled bodies that were similar in morphology to

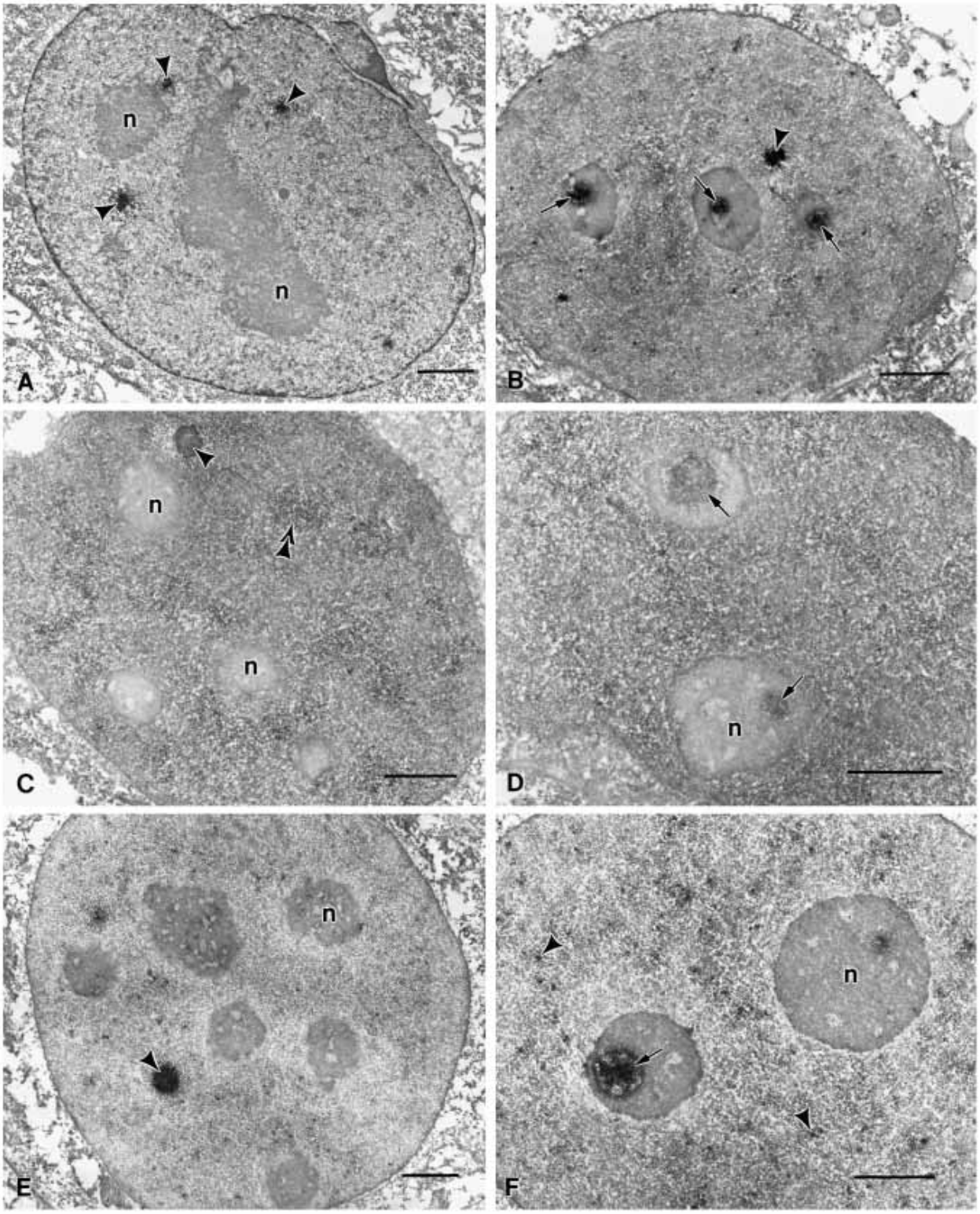

Fig. 5. Pre-embedding immunoperoxidase electron microscopic localization of coilin (A,B,E,F) and Sm (C,D) in HEp-2 cells (A), or in T47D (B-D) or MCF-7/AZ (E,F) breast cancer cells. In HEp-2 cells (A), coilin was localized in nuclear bodies (arrowheads) throughout the nucleoplasm but never within nucleoli (n). In T47D cells (B), coilin was localized in nuclear bodies in the nucleoplasm (arrowhead) and in nucleoli (arrows). Sm staining (C,D) was localized to nuclear bodies in the nucleoplasm (arrowhead in C) and in nucleoli (arrows in D), and throughout the nucleoplasm in patches (double arrowheads in C) excluding nucleoli (n). Note that nuclear bodies in the nucleoplasm stain more intensely for Sm than do nuclear bodies in nucleoli. MCF-7/AZ cells also had coilin-staining nuclear bodies in the nucleoplasm (arrowhead in E) and within nucleoli (arrow in F). Note the small patches of coilin staining (arrowheads in F) distributed throughout the nucleoplasm. Bar, (A-F), 2.0 μm.

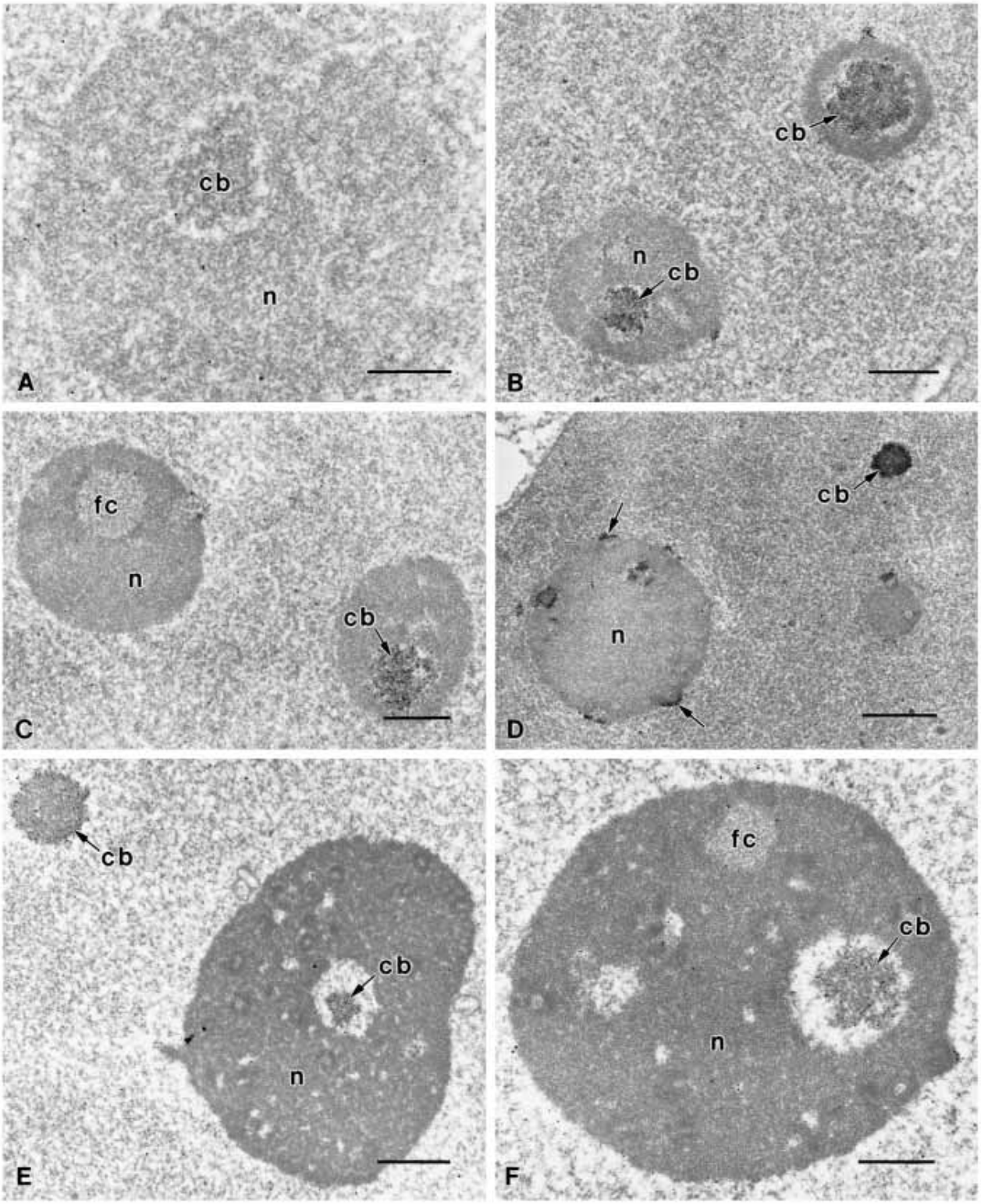

Fig. 6. Pre-embedding silver-enhanced Nanogold immunoelectron microscopic localization of p80-coilin in T47D (A-D) or MCF-7/AZ (E,F) breast cancer cells. In negative control cells with no primary antibody (A), few silver-enhanced gold particles were observed in nucleoli (n) or coiled bodies (cb). In T47D cells (B-D), coilin was localized primarily to coiled bodies (cb) within nucleoli (n) or in the nucleoplasm (D). Outside of coiled bodies in the nucleolus, coilin was not present in fibrillar centers (fc) or in other structural components of the nucleolus. Occasionally, coilin was localized to the surface of the nucleolus (arrows in D) in structures that were not identifiable as coiled bodies. Note the very heavy labeling of nucleoplasmic coiled bodies (D) compared to nucleolar coiled bodies (B,C). The distribution of coilin in MCF-7/AZ cells was similar to that of T47D cells in the heavier labeling of nucleoplasmic coiled bodies (E) compared to those in the nucleolus (E,F) and the absence of label in fibrillar centers (F) or elsewhere in the nucleolus (E,F). Bars: (A), 0.5 μm; (B,C,E), 1.0 μm; (D,F), 2.0 μm.

two questions, we performed pre-embedding immunogold electron microscopy in conjunction with silver enhancement of ultrasmall 1.4 nm gold particles covalently linked to Fab' fragments (Nanogold from Nanoprobes, Inc.) as the secondary detecting reagent for the localization of p80-coilin in T47D (Fig. 6A-D) and MCF-7/AZ cells (Fig. 6E,F). In negative controls with no primary antibody (Fig. 6A), few silver-enhanced gold particles were observed over nucleoli or coiled bodies. In T47D cells (Fig. 6B-D), coilin was localized primarily to coiled bodies within nucleoli or in the nucleoplasm. Gold particles were distributed evenly throughout both nucleolar and nucleoplasmic coiled bodies, and labeling of nucleoplasmic bodies was much heavier than that of nucleolar bodies (compare Fig. 6D with B or C). Presumably this labeling pattern is a reflection of the even distribution of p80-coilin throughout the coiled body, and may indicate that coilin is a structural component, comprising the fibrils of coiled bodies. Outside of coiled bodies in the nucleolus, coilin was not detected in fibrillar centers or in any other structural components of the nucleolus (Fig. 6B,C). Occasionally, coilin was localized to the surface of the nucleolus in patches of staining that were not identifiable as coiled bodies (arrows in Fig. 6D). The distribution of p80-coilin in MCF-7/AZ cells was similar to that of T47D in the heavier labeling of nucleoplasmic coiled bodies (Fig. 6E) compared to nucleolar ones (Fig. 6E,F) and the absence of label in fibrillar centers (Fig. 6F) or elsewhere in the nucleolus.

Table 1 summarizes the quantitation of silver-enhanced Nanogold labeling of T47D cells with no primary antibody (control), with anti-coilin and with anti-fibrillarin. Results were expressed as number of silver-enhanced gold particles/μm^2 of surface area over cytoplasm, nucleoplasm, nucleolus, nucleoplasmic coiled bodies and nucleolar coiled bodies. Negative controls varied from a low concentration of 7.7 over the cytoplasm and nucleolus to a high of 17.7 over nucleolar coiled bodies. No nucleoplasmic coiled bodies were analyzed with negative controls. Labeling with anti-coilin was at background concentration over the nucleolus at 17.5, somewhat above background for the cytoplasm at 22.4 and for the nucleoplasm at 33.4, and heavily concentrated over nucleolar coiled bodies at 1,115 gold particles/μm^2. Labeling was so intense over nucleoplasmic coiled bodies that it was impossible to quantitate the amount. Labeling concentration for the nucleoplasm was somewhat under-represented since there were small concentrated patches of gold particles distributed throughout the nucleoplasm, much like the peroxidase labeling of the nucleoplasm with anti-coilin in Fig. 5F.

Nucleolar coiled bodies are not colocalized with fibrillar centers and they do not contain fibrillar center proteins, RNA polymerase I or NOR-90/UBF

Since nucleolar coiled bodies are round, within the size range for large fibrillar centers, and morphologically somewhat similar in appearance to them, we performed immunoelectron microscopy on nucleoli from T47D cells to determine whether the coilin-containing nucleolar bodies also contained protein components of fibrillar centers or other nucleolar domains. Marker antibodies to fibrillarin were used to identify the nucleolar dense fibrillar component (Fig. 7A,B), antibodies to RNA polymerase I for identification of fibrillar centers (Fig. 7C), and antibodies to NOR-90/UBF for identification of both fibrillar centers and dense fibrillar component (Fig. 7D). Nucleolar coiled bodies contained fibrillarin but no appreciable amounts of RNA polymerase I or NOR-90/UBF. As expected, fibrillarin was also concentrated in the nucleolar dense fibrillar component (Fig. 7A,B), RNA polymerase I was localized exclusively to fibrillar centers (Fig. 7C), and NOR-90/UBF was confined to fibrillar centers and the surrounding dense fibrillar component (Fig. 7D). Somewhat to our surprise, fibrillarin labeling was consistently more concentrated in nucleoplasmic coiled bodies (see inset to Fig. 7B and Table 1) compared to nucleolar coiled bodies. Table 1 illustrates this point by showing that the concentration of fibrillarin in nucleoplasmic coiled bodies was almost 5 times that in nucleolar coiled bodies (632 compared to 138).

Coilin does not appear altered in breast cancer cells compared to other cell types

Since this is the first report of a cell line with p80-coilin and coiled bodies in the nucleolus, we performed western blotting on a number of different human cell lines with and without nucleolar coiled bodies (Fig. 8A) to determine if there was some easily detectable alteration in the migration or amount of coilin that could account for this novel finding. As detected by antibody binding, coilin was present as an 80 kDa singlet or doublet (arrowhead in Fig. 8A) in every cell line tested, whether or not it had nucleolar coiled bodies. No anomalously migrating bands were detected and the relative amounts of coilin present in each cell line were highly variable.

In this study, Sm staining was also reported to be localized in the nucleolus for the first time in any mammalian cell line. The obvious explanation is that Sm proteins are known to be localized to coiled bodies, whether they are in the nucleoplasm

Table 1. Quantitation of Nanogold pre-embedding immunoelectron microscopy

	Silver-enhanced gold particles/μm^2±s.e.m.				
	Cyto[a]	Nu[b]	No[c]	Nu CBs[d]	No CBs[e]
Control[f]	7.7±1.2	14.3±3.8	7.7±2.3	–	17.7±3.1
Anti-coilin	22.4±4.5	33.4±6.0	17.5±2.8	tntc[g]	1,115±70
Anti-fibrillarin	–	–	–	632±38	138±39

[a]Cytoplasm.
[b]Nucleoplasm, excluding nucleoli and nucleoplasmic coiled bodies.
[c]Nucleoli, excluding nucleolar coiled bodies.
[d]Nucleoplasmic coiled bodies.
[e]Nucleolar coiled bodies.
[f]Negative control, no primary antibody.
[g]Too numerous to count.

or the nucleolus. Alternatively, if the human anti-Sm autoantibody used in our study also contained anti-coilin antibodies, this could explain the 'apparent' Sm staining in nucleolar coiled bodies as well. Fig. 8B shows the specificity of our Sm autoantibody for the major 28 kDa BB' Sm protein (arrowhead in Fig. 8B) in a number of different human cell lines, and just as importantly, the lack of staining for coilin in the 80 kDa molecular mass range (arrowhead in Fig. 8A).

DISCUSSION

Coiled bodies in the nucleolus

Why are coiled bodies found in nucleoli of certain human breast cancer cell lines? A number of possibilities may explain this phenomenon. Coiled bodies could have arisen in the nucleolus due to some mutational event. For example, if the gene for p80-coilin was altered by translocation, recombina-

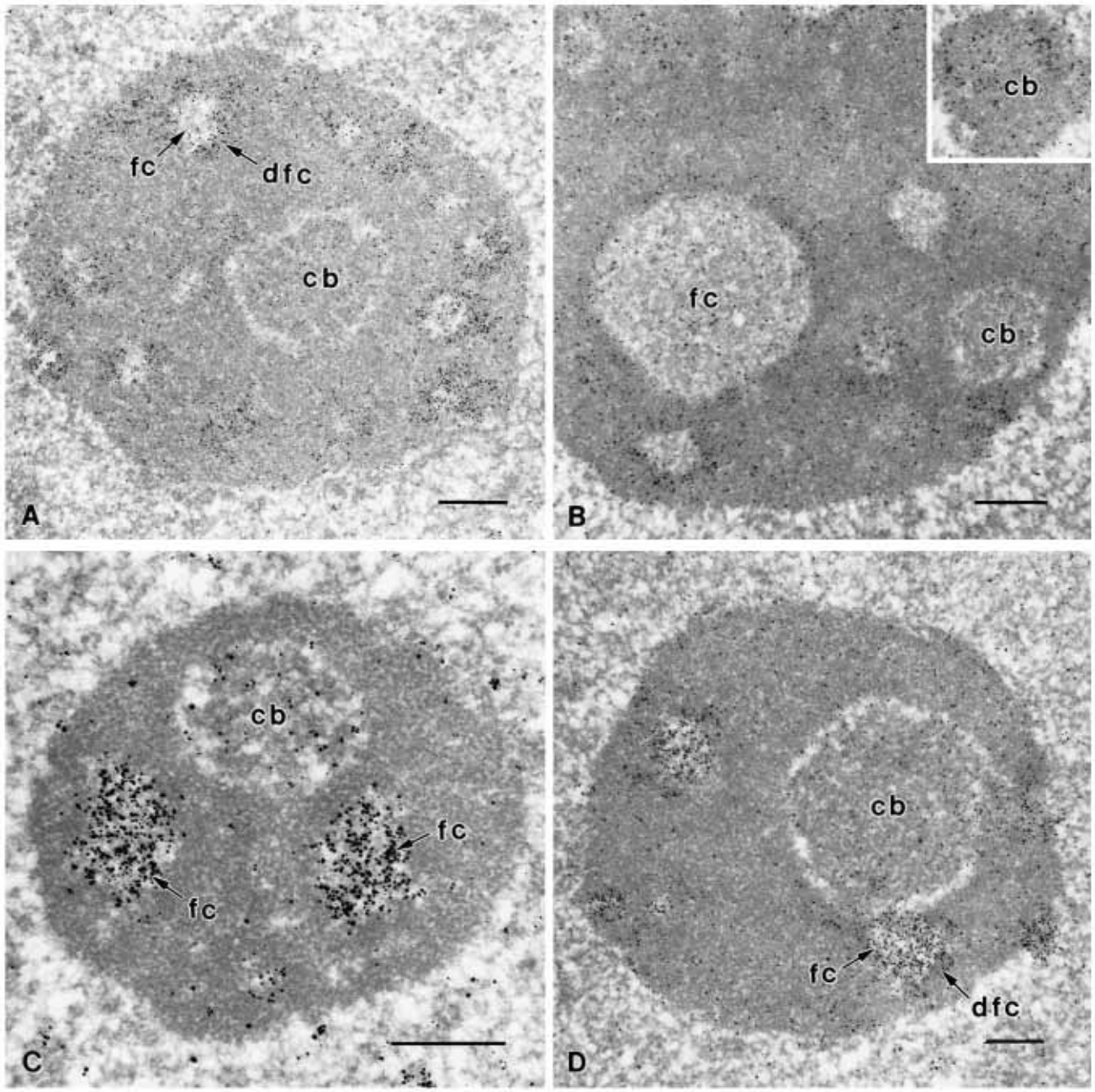

Fig. 7. Pre-embedding silver-enhanced Nanogold immunoelectron microscopic localization of fibrillarin (A,B), RNA polymerase I (C), and NOR-90 (D) in T47D cells. In T47D cells, fibrillarin (A,B) was localized in the dense fibrillar component (dfc) of the nucleolus surrounding small fibrillar centers (fc), and less intensely in nucleolar coiled bodies compared to those found in the nucleoplasm (inset to B). RNA polymerase I (C) was localized primarily to fibrillar centers (fc), with little labeling of nucleolar coiled bodies (cb). NOR-90 (D) was localized to both nucleolar fibrillar centers (fc) and the dense fibrillar component (dfc), with no labeling of nucleolar coiled bodies (cb). Bars: (A-D), 0.5 μm.

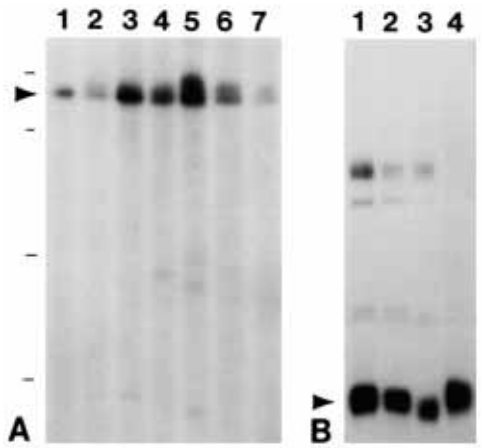

Fig. 8. Western blotting of whole cell extracts with human autoantibodies to p80-coilin (A) or to Sm proteins (B). A 25 μg sample of protein was loaded in each lane. (A) Lane 1, MOLT-4; lane 2, HEp-2; lane 3, HBL-100; lane 4, T47D; lane 5, MCF-7; lane 6, MCF-7/AZ; lane 7, MCF-7/6. By western blotting, human autoantibody to p80-coilin recognized a major band (and sometimes a doublet) at approximately 80 kDa (arrowhead) in all cell lines tested. (B) Lane 1, MOLT-4; lane 2, HEp-2; lane 3, T47D; lane 4, MCF-7. Human anti-Sm recognized the major BB′ band at 28 kDa (arrowhead). Note the lack of anti-coilin reactivity in the 80 kDa region of the gel with this particular anti-Sm serum.

tion or insertional mutagenesis so that a nucleolus localization signal was subsequently attached to p80-coilin, then this normally occurring nucleoplasmic protein could be targeted to the nucleolus, where a coiled body could then form. Initially, we tested for an altered form of p80-coilin by western blotting, but no aberrant proteins were detected. Of course the possibility still exists that a minor alteration in the protein is responsible for its abnormal localization, and this may only be detectable at the level of the gene.

A minor variation of the mutation theory may relate to tumor heterogeneity and clonal selection. Since the human breast cancer cell lines tested were all derived as secondary metastatic pleural effusions from primary breast carcinomas (Soule et al., 1973; Keydar et al., 1979; Bracke et al., 1991; Coopman et al., 1991), the original tumors may have contained populations of cells with and without nucleolar coiled bodies. When the original cells were cultured, some of each type may have been present and, depending upon the selective pressures existing in culture, cells of one type or the other may have been selected for, resulting in cells enriched for or depleted in nucleolar coiled bodies. In this scenario, the occurrence (or absence) of nucleolar coiled bodies in different breast cancer cell lines may be considered an in vitro artifact, not at all reflective of the original breast carcinoma. Such a course of events could explain why MCF-7 cells do not appear to have nucleolar coiled bodies (or at least have very few) and the MCF-7-derived cell lines MCF-7/AZ and MCF-7/6 do. In fact, heterogeneity in MCF-7 cells has been reported (Seibert et al., 1983; Resnicoff et al., 1987).

Alternatively, the presence of coiled bodies in the nucleolus may be physiologically or cell cycle-related, as shown previously for nucleoplasmic coiled bodies (Andrade et al., 1993). Even in cell lines that possess nucleolar coiled bodies, only a certain percentage of cells at any one time have coiled bodies in their nucleoli. Therefore, a combination of the above possibilities may exist such that a mutational event is necessary for nucleolar coiled bodies to occur but their frequency may be transitory, dependent upon other physiological factors such as hormone responsiveness or stage of the cell cycle. Only further experimentation will allow us to distinguish between these, and other, possibilities.

At present, we are unaware of other cell lines that have coiled bodies in the nucleolus. Should the finding of coiled bodies in nucleoli of breast cancer cells be an isolated case, then this observation may be diagnostically or clinically useful in relationship to malignant transformation of breast tissue.

The relationship between coiled bodies and the nucleolus

Several lines of evidence indicate a relationship between coiled bodies and the nucleolus. Coiled bodies are often found in close proximity to nucleoli and have even been reported to 'bud' from their surface (Raska et al., 1990a; Lafarga et al., 1991). In addition, coiled bodies contain what were previously thought to be nucleolus-specific proteins: fibrillarin and the NOR silver staining protein (Raska et al., 1990a). Therefore, coiled bodies may be derived structurally from the nucleolus; thereby containing some nucleolar components (Raska et al., 1990a, 1991), and yet they may play an active role in metabolism of pre-mRNA in the nucleoplasm as well (Raska et al., 1991; Carmo-Fonseca et al., 1992, 1993; Lamond and Carmo-Fonseca, 1993; Matera and Ward, 1993). The nucleolar derivation of coiled bodies would explain how fibrillarin, as part of the nucleolar matrix structural skeleton (Ochs and Smetana, 1991), could be located in both the nucleolus and coiled bodies. The fact that coiled bodies in the nucleolus are smaller and contain less coilin, Sm, and fibrillarin than nucleoplasmic coiled bodies (this paper) may be considered indirect proof that coiled bodies form in the nucleolus, migrate to the nucleoplasm, and then grow or fuse to form bigger nucleoplasmic coiled bodies to which more coilin, Sm and fibrillarin can be added. Even though we tend to favor this possibility, we have no direct proof. Conceivably, coiled bodies could be fusing with the nucleolus, and reports of budding from the nucleolar surface may be coiled bodies in the act of incorporation into the nucleolus. This could explain why the nucleolus does not normally stain for p80-coilin - it may be diluted on fusion of coiled bodies with the nucleolus and be beyond the sensitivity of present detection methods. Clearly, more studies need to be done before we can determine the mechanism(s) of coiled body formation.

Coiled bodies and cancer

It may not be accidental that nucleolar coiled bodies have been first observed in breast cancer cells. Some of the earliest reports of nuclear bodies actually derived from the ultrastructural examination of cells from human tumors (Bouteille et al., 1967; Brasch and Ochs, 1992). Specific to coiled bodies, Spector et al. (1992) compared the percentage of cells with coiled bodies in human cell lines of various degrees of transformation. They noted the lowest percentage of cells with coiled bodies in contact-inhibited cells of defined passage, more in immortal cells, and the highest percentages in cells

fully transformed by adenovirus infection. Therefore, a correlation existed between the degree of transformation and the presence of coiled bodies.

In addition to cancer cells, hormone-responsive cells are also known to have many different types of nuclear bodies that are indicative of cellular hyperactivity (see Brasch and Ochs, 1992, for review). The breast cancer cell lines T47D and MCF-7 used in our studies were reported to have receptors for estradiol and progesterone (Vic et al., 1982), vitamin D_3 (Sher et al., 1981; Freake et al., 1981) and calcitonin (Lamp et al., 1981). Whether or not the hormone responsiveness of these cell lines is related to the presence or nucleolar localization of coiled bodies will be a topic for future investigation.

The study of MCF-7 and its derivative cell lines that have nucleolar coiled bodies may provide excellent models for investigating coiled bodies and their relationship to the nucleolus and cancer. Already, analyses of nucleoli in breast cancer tissues has been employed clinically to grade tumors (Helpap, 1989) and the nucleolar Ag-NOR staining technique is being used in the pathological examination of many different types of cancers, including those of the breast (Smith and Crocker, 1988). Along these same lines, the study of coiled bodies in breast carcinomas, and other cancer tissues, may also prove to be clinically relevant.

Novel functions for the nucleolus

The discovery of coiled bodies and spliceosome-associated Sm proteins in the nucleolus may be indicative of novel functions for what was always assumed to be a monofunctional organelle, responsible only for the synthesis and packaging of preribosomal RNA. Interestingly, Potashkin et al. (1990) reported Sm staining in the yeast nucleolus, suggesting that the 'primitive' nucleolus may have at one time combined preribosomal RNA and premessenger RNA processing events. Other reports also reflect what may be novel nucleolar functions. For example, during the cell cycle centromeres are transiently associated with the nucleolus in what may be a physiological association (Ochs and Press, 1992), and c-*myc* RNA transcripts were recently localized to the nucleolus, leading to the speculation that the nucleolus may be involved in the maturation or transport of some Pol II gene transcripts (Bond and Wold, 1993). A number of studies also indicate a role for the nucleolus in viral infections (Walton et al., 1989), most recently in regard to nucleolar localization of viral proteins *rev* and *tat* of the HIV virus (Cochrane et al., 1990; Siomi et al., 1990) and *rex* of HTLV I (Nosaka et al., 1989; Kalland et al., 1991). The significance of these findings, as with nucleolar coiled bodies, is not yet apparent but they may be indicative of a multifunctional nucleolus.

Technical comments

The ability of Nanogold to penetrate into the interior of the nucleolus to successfully label nucleolar coiled bodies, with subsequent silver enhancement to an arbitrary size gold particle, makes this probe highly desirable for pre-embedding immunoelectron microscopy and comparable to peroxidase in its penetrating ability. In addition, it has the advantage of particulateness and quantifiability, as well as not obscuring the object of interest, as does the peroxidase reaction product. Compared to post-embedding immuno-EM on thin sections of LR White-embedded cells (data not shown), the pre-embedding method afforded almost 3 times heavier labeling density with somewhat lower background. Labeling of coiled bodies in the interior of the nucleolus, which is itself very dense and often located in the most interior region of the nucleus, is perhaps one of the most severe challenges for a pre-embedding technique. Therefore, we recommend the pre-embedding silver-enhanced Nanogold technique for many of the applications that in the past may have relied exclusively on post-embedding procedures with colloidal gold probes.

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