Assembly of snRNP-containing Coiled Bodies Is Regulated in Interphase and Mitosis—Evidence that the Coiled Body Is a Kinetic Nuclear Structure

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Abstract. Coiled bodies (CBs) are nuclear organelles in which splicing snRNPs concentrate. While CBs are sometimes observed in association with the nucleolar periphery, they are shown not to contain 5S or 28S rRNA or the U3 snoRNA. This argues against CBs playing a role in rRNA maturation or transport as previously suggested. We present evidence here that CBs are kinetic structures and demonstrate that the formation of snRNP-containing CBs is regulated in interphase and mitosis. The coiled body antigen, p80 coilin, was present in all cell types studied, even when CBs were not prominent. Striking changes in the formation of CBs could be induced by changes in cellular growth temperature without a concomitant change in the intracellular p80 coilin level. During mitosis, CBs disassemble, coinciding with a mitotic-specific phosphorylation of p80 coilin. Coilin is shown to be a phosphoprotein that is phosphorylated on at least two additional sites during mitosis. CBs reform in daughter nuclei after a lag period during which they are not detected. CBs are thus, dynamic nuclear organelles and we propose that cycling interactions of splicing snRNPs with CBs may be important for their participation in the processing or transport of pre-mRNA in mammalian cells.

THE identification of subcellular organelles and the characterization of metabolic activities which take place within them are major goals of cell biology. In the nucleus, only one clear example of a functional compartment has so far been characterized in any detail, i.e., the nucleolus. The nucleolus is a membraneless nuclear organelle in which rRNA is transcribed and processed and ribosomal subunits assembled (reviewed by Warner, 1990; Scheer and Benavente, 1990). Nucleolar structures assemble around chromosomal sites containing the rDNA repeat regions and recruit, by a poorly understood mechanism, both the panoply of transcription and processing factors required for production of mature rRNA and the additional protein and RNA components needed for ribosome assembly. When cells divide, both rRNA synthesis and ribosome assembly is halted and in prophase the nucleolus is disassembled. During telophase, nucleoli reform around the rDNA repeats in the chromosomes of the daughter nuclei.

Considerably less is known about the nuclear organization of the many factors required to generate and export mRNA, or about their functional relationship to specific nuclear substructures. Yet mRNA formation, particularly in higher eukaryotes, is a multistep process with most primary transcripts requiring extensive processing, including both splicing and 3′polyadenylation, to yield mature mRNA. Some mechanism must also exist to selectively export mRNA but not excised introns. The major subunits of splicing complexes are the U1, U2, U4/U6, and U5 snRNPs, all of which are required for both spliceosome assembly and splicing (reviewed by Maniatis and Reed, 1987; Steitz et al., 1988; Guthrie and Patterson, 1988; Lührmann et al., 1990). These splicing snRNPs, together with non-snRNP splicing factors, must locate pre-mRNAs in the nucleus, assemble into functional complexes and, when splicing is completed, must be recycled to splice new transcripts. The mRNA products must also be separated from the introns and transported to the cytoplasm. At present, it is not known how these various processes occur in vivo, how they are integrated with transcription and polyadenylation, or how they are controlled during the cell division cycle.

Many previous studies have analyzed the nuclear distribution of splicing snRNPs in mammalian cells by indirect immunofluorescence using autoimmune and mAbs specific for either snRNP proteins, or the snRNA-specific 2,2,7 trimethyl guanosine cap structure (m3G-Cap) (Northway et al., 1972; Deng et al., 1981; Reuter et al., 1984; Spector, 1984; Nymmann et al., 1986; Verheijen et al., 1986; Habets et al., 1989). These antibodies show widespread nucleoplasmic staining with additional concentrations of snRNPs in 20–50 punctate structures. We have recently analyzed the distributions of each individual species of splicing snRNP in mammalian cells using a combination of in situ hybridization with antisense probes to detect snRNA sequences and parallel double labeling with antibodies to detect cognate snRNP

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proteins (Carmo-Fonseca et al., 1991*a*,*b*; Carmo-Fonseca et al., 1992). A major conclusion from these studies was that the punctate structures seen by immunofluorescence using anti-snRNP probes represent at least two distinct classes of intranuclear snRNP compartments, coiled bodies (CBs)[1] and interchromatin granules. A similar conclusion was reported by Huang and Spector (1992). CBs and interchromatin granules represent metabolically distinct snRNP compartments because treatment of cells with drugs which inhibit transcription decreases the concentration of splicing snRNPs in CBs but increases their concentration in interchromatin granules (Carmo-Fonseca et al., 1992). CBs are detected in the nuclei of most mammalian cells as bright foci of splicing snRNPs (Carmo-Fonseca et al., 1991*a*, 1992; Raska et al., 1991; Spector et al., 1992). By microinjecting fluorochrome-coupled antisense probes specific for snRNAs into the nuclei of unfixed, living cells it was also demonstrated that these foci contain high concentrations of splicing snRNPs in vivo (Carmo-Fonseca et al., 1991*b*). As well as splicing snRNPs, CBs contain both the 65- and 35-kD subunits of the non-snRNP splicing factor U2AF (Zamore and Green, 1991; Carmo-Fonseca et al., 1991*a*; Zhang et al., 1992). However, another essential splicing factor, SC-35 (Fu and Maniatis, 1990), is not detected in CBs (Carmo-Fonseca et al., 1991*b*; Raska et al., 1991; Spector et al., 1991; Carmo-Fonseca et al., 1992; Huang and Spector, 1992). Instead, SC-35 has been localized to interchromatin granules and perichromatin fibrils (Spector et al., 1991). The functional roles of these separate nuclear snRNP compartments remain to be determined.

The CB was first identified as a distinct nuclear structure at the beginning of the century, by Ramon y Cajal, who called it the "accessory body" and showed using light microscopy that it stained strongly with silver in neuronal tissue (Ramon y Cajal, 1903). This structure was later reidentified by electron microscopy and renamed the "coiled body" (Hardin et al., 1969; Monneron and Bernhard, 1969). Subsequent EM studies have detected CBs in the nuclei of both plant and mammalian cells, showing that it is an evolutionarily conserved structure (Moreno Diaz de la Espina et al., 1980, 1982; Seite et al., 1982; Schulz, 1990). It was also observed by immunoelectron microscopy that anti-Sm antibodies label CBs, indicating that they contain snRNPs (Fakan et al., 1984). A major advance in studying the CB has been the recent characterization of a novel class of autoimmune antibodies which specifically stain CBs and identify a CB-specific protein, termed p80 coilin (Raska et al., 1990, 1991; Andrade et al., 1991).

In this study we present evidence that the formation of snRNP-containing CBs is a kinetic process which is regulated during interphase and mitosis. The CB antigen, p80 coilin, is shown to be a phosphoprotein that is specifically phosphorylated on at least two additional sites during mitosis. Coilin is detected in all cell types studied but only accumulates in prominent CBs under specific metabolic conditions.

1. *Abbreviation used in this paper*: CBs, coiled bodies.

Materials and Methods

Cell Culture

HeLa cells were grown as previously described (Carmo-Fonseca et al., 1992). Enrichment for rounded-up mitotic cells was achieved by mechanical shake off. HeLa cells were arrested in mitosis by adding nocodazole to the medium for 20 h at a final concentration of 0.4 μg/ml. To obtain a synchronous population of interphase HeLa cells arrested in G1-S phase, hydroxyurea was added to the medium for ~20 h at a final concentration of 10 mM. Primary rat hippocampal neurons were isolated and cultured as described by Dotti and Simons (1990).

Immunofluorescence and In Situ hybridization

Cells were grown on glass coverslips and harvested at 70–80% confluency. Coverslips with attached cells were washed in PBS and incubated with 3.7% paraformaldehyde in 100 mM NaCl, 300 mM sucrose, 10 mM Pipes pH 6.8, 3 mM $MgCl_2$, 1 mM EGTA (i.e., CSK buffer, Fey et al., 1986) for 10 min at room temperature. After fixation, the cells were permeabilized with either 0.5% Triton X-100 in CSK buffer or 0.2% SDS in 20 mM Tris pH 7.4, 30 mM NaCl, 2.5 mM EDTA for 15 min at room temperature.

Immunofluorescence and in situ hybridization with biotinylated 2′-O-alkyl oligoribonucleotides were performed as previously described (Carmo-Fonseca et al., 1992). Splicing snRNPs were labeled with the anti-Sm mAb Y12 (Pettersson et al., 1984). The CB antigen, p80 coilin, was detected using rabbit antibodies raised against a β-galactosidase-coilin fusion protein and patient autoantiserum "Sh" (Andrade et al., 1991). Fibrillarin was labeled using the mAb 72B9 and also using monospecific human autoantisera and affinity-purified rabbit antibodies raised against a carboxy-terminal peptide of human fibrillarin (Janssen et al., 1991). Biotinylated 2′-O-alkyl oligoribonucleotide probes were synthesized as described by Sproat and Lamond (1991). Sequences of the probes used were: (*a*) anti-U3 snRNA, 5′-UUUCIIUICUCdT-3′; (*b*) anti-28S rRNA, 5′AIAICCAAUCCUUAUdT-3′; and (*c*) anti-5S rRNA, 5′-CIIUAUUCCAIICIdT-3′. All probes were made of 2′-O-allyl RNA (Iribarren et al., 1990) with an additional 3′ terminal deoxythymidine. The nucleotides used were uridine (U), adenosine (A), cytosine (C), and inosine (I). Each probe carried two tandem biotin residues at both the 5′ and 3′ ends of the antisense sequence (Pieles et al., 1990).

Electron Microscopy

For immunoelectron microscopy cells were grown on plastic Petri dishes. The cells were briefly extracted with 0.1% Triton X-100 in CSK buffer containing 0.1 mM PMSF for 30 s on ice and fixed in 3.7% paraformaldehyde in CSK buffer for 30 min at room temperature. After fixation cells were further extracted with 0.5% Triton in CSK for 30 min, washed 3× for 10 min with PBS and rinsed twice with Tris buffer (20 mM Tris-HCl pH 8.2, 0.5 M NaCl, 0.05% Tween 20 and 0.1% BSA). The cells were incubated with rabbit anticoilin antibody diluted 1:50 in Tris buffer for 2 h at room temperature, washed 3× for 10 min in the same buffer and incubated with goat anti-rabbit IgG coupled to 5 nm gold particles (Amersham Corp., Arlington Heights, IL) for 2 h at room temperature. After washing in Tris buffer, the samples were rinsed 3× with PBS, fixed in 2% glutaraldehyde in PBS for 10 min, post-fixed in 0.05% OsO_4, contrasted with 0.2% tannic acid and 0.5% uranyl acetate/1% phosphotungstic acid and embedded in Epon as described by Langanger et al. (1984). Samples were examined with a Philips EM 301 electron microscope operated at 80 kV.

Confocal Microscopy

Confocal microscopy was carried out using the EMBL compact confocal microscope (CCM) developed by Stelzer (Stelzer et al., 1992). Excitation wavelengths of 488 nm (fluorescein fluorescence) and 529 nm (Texas red fluorescence) were selected from an Argon-ion laser. For double labeling experiments, each fluorochrome was independently recorded. Pseudocolored images of both signals were generated and superimposed. Images were photographed on Fujichrome 100 or Kodak Tmax 100 film using a Polaroid Freeze Frame Recorder.

SDS-PAGE and Immunoblotting

Cells were harvested by scraping with a rubber policeman and lysed by boil-

ing for 5 min in SDS-PAGE sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol and 0.01% bromophenol blue). Proteins were separated by SDS-PAGE on 8% acrylamide gels and transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). The membranes were blocked and washed with 2% nonfat milk powder in PBS. The blots were incubated overnight with primary antibodies diluted in washing buffer, washed and incubated for 1 h with secondary antibodies conjugated to alkaline phosphatase (BioRad Labs, Richmond, California).

Immunoprecipitation and Alkaline Phosphatase Digestion

Cells were solubilized in lysis buffer (0.2% SDS, 1% NP40, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.1 mM PMSF and 10 μg/ml of each of the following protease inhibitors: chymostatin, leupeptin, antipain and pepstatin) for 30 min at 4°C on a rotating shaker. Immunoprecipitation was performed using protein A Sepharose CL-4B (Pharmacia, Uppsala, Sweden) as described by Brändli et al. (1990). For alkaline phosphatase treatment the immunoprecipitated samples were resuspended in CIP buffer (50 mM Tris-HCl pH 9.0, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1 mM spermidine) containing 0.1 mM PMSF and 10 μg/ml of each of the following protease inhibitors: chymostatin, leupeptin, antipain, and pepstatin. Approximately 10 U of enzyme (alkaline phosphatase from bovine intestinal mucosa, P-7915 Sigma Chemical Co., St. Louis, MO) was added to 20 μl of protein A-Sepharose slurry. As a control, 10 U of enzyme were added in the presence of 100 mM β-glycerophosphate. The samples were incubated for 30 min at 30°C, washed twice with 10 mM Tris pH 4.5 and boiled for 5 min in SDS-PAGE sample buffer.

Metabolic Labeling

Interphase (hydroxyurea treated) and mitotic (nocodazole treated) cells plated in flasks were washed twice and incubated for 30 min at 37°C with phosphate-free medium (GIBCO BRL, Gaithersburg, MD) buffered with 25 mM Hepes (pH 7.4) in the presence of nocodazole or hydroxyurea. Radiolabeling was performed by adding to the medium 0.5 mCi/ml [^{32}P]orthophosphate (Phosphor-32, Amersham, Buckinghamshire, UK) and incubating for 3 h at 37°C. The cells were then rinsed twice with ice-cold PBS, lysed and p80 coilin protein isolated by immunoprecipitation as described above.

Phosphoamino Acid Analysis

Phosphoamino acid analysis was performed using the procedure described by Kamps and Sefton (1988). Proteins labeled in vivo with [^{32}P]orthophosphate as described above were transferred to Immobilon-P (Millipore Corp., Milford, MA) membranes, identified by autoradiography, excised and washed several times in water. The membrane pieces were then incubated for 70 min at 110°C in 6N HCl (Pierce Chemical Co., Rockford, Illinois) in a sealed Eppendorf tube containing nitrogen. The insoluble material was removed and the sample was dried under vacuum. The dried material was resuspended in pH 3.5 buffer (pyridine:glacial acetic acid:water, 4:40:756 by volume). The sample was loaded on a thin layer cellulose chromatography plate (Eastman Kodak Co., Rochester, NY) and run at 750 V for 45 min. As markers, 5 μg of each unlabeled phosphoamino acid (Sigma Chemical Co.) were mixed together and loaded either separately or added to the samples. The unlabeled phosphoamino acids were visualized by spraying the plate with 0.2% ninhydrin in acetone.

Two-dimensional Tryptic Mapping

Two-dimensional analysis of tryptic peptides was performed according to the procedure described by Luo et al. (1990). Coilin was labeled in vivo with [^{32}P]orthophosphate in HeLa cells blocked with drugs either in interphase or in mitosis, isolated by immunoprecipitation and transferred to nitrocellulose membrane as described above. Pieces of nitrocellulose membrane containing the coilin protein were washed with water and then soaked in 0.5% polyvinylpyrrolidone (PVP-360, Sigma Chemical Co.) in 100 mM acetic acid for 30 min at 37°C. The membrane pieces were then washed 5× with water, 2× with 0.05 M ammonium bicarbonate (prepared fresh) and then digested with 10 μg TPCK Trypsin (Worthington Biochem. Corp., Freehold, NJ) in the same buffer for 4 h at 37°C. A second aliquot of 10 μg TPCK Trypsin was then added and incubation continued for a further 4 h at 37°C. The tube was then filled with water and the sample dried under vacuum. The sample was loaded on a thin layer cellulose chromatography plate (Eastman Kodak Co.) and two-dimensional peptide separation performed using electrophoresis at pH 8.9 in the first dimension and ascending chromatography in the second dimension as described by Hunter and Sefton (1980).

Results

Coiled Body Size and Nuclear snRNP Distributions Are Temperature Dependent

The size of CBs and the amount of splicing snRNPs they contain were observed to change in the interphase nuclei of cells grown at different temperatures, indicating that the CB is a dynamic structure (Fig. 1). In HeLa cells grown at 37°C, prominent snRNP foci are detected with anti-Sm antibodies which colocalize with the CBs revealed by anticoilin antibodies (Fig. 1, *A* and *D*). However, at 32°C CBs are larger and the amount of snRNPs in CBs has increased (Fig. 1, *B* and *E*). In contrast, at 39°C, CBs are very small and can hardly be detected as discrete structures by anti-snRNP antibodies (Fig. 1, *C* and *F*). The overall pattern of snRNP staining in the nucleus is also markedly different at 32 and 39°C. At the lower temperature, snRNP staining is widespread, similar to 37°C, while at 39°C it is predominantly concentrated in 20–50 nucleoplasmic speckles (Fig. 1, *A–C*). Double labeling studies show that these speckled structures correspond to interchromatin granules (data not shown). The bulk movement of splicing snRNPs into interchromatin granule speckles and out of the widespread nucleoplasmic and CB compartments was previously observed when cells were treated with the transcription inhibitors actinomycin D or α-amanitin (Carmo-Fonseca et al., 1992). The speckled snRNP distribution observed at 39°C may therefore result from a reduced level of transcription caused by thermal stress.

Coiled Bodies and p80 Coilin Are Present in Primary Fibroblasts

It was recently reported that snRNP foci are not present in primary human fibroblasts of defined passage number and consequently the authors argued that CBs were unlikely to play an essential role connected with pre-mRNA processing (Huang and Spector, 1992). In a subsequent study, the same group reported that CBs were actually present in primary fibroblasts, but only in 2–3% of the cells in a population (Spector et al., 1992). Given the sensitivity of CB formation to cellular growth conditions as shown above, we decided to reinvestigate this issue. In agreement with the previous studies, we observed that anti-snRNP antibodies show a predominantly speckled staining pattern in primary human fibroblasts grown at 37°C (Fig. 1 *G*), resembling the HeLa pattern seen at 39°C (Fig. 1 *C*). A similar speckled pattern was seen in fibroblasts grown at 39°C (Fig. 1 *I*). However, in fibroblasts grown at 32°C, the snRNP staining is more widespread in the nucleoplasm and foci are detected (Fig. 1 *H*). Double labeling with anti-snRNP and anticoilin antibodies confirms that these snRNP foci are CBs, which are readily detected in over 90% of the cells examined (Fig. 1, *J* and *K*). Interestingly, coilin staining in fibroblasts grown at 29°C not only revealed prominent CBs, but also nucleolar "caps"

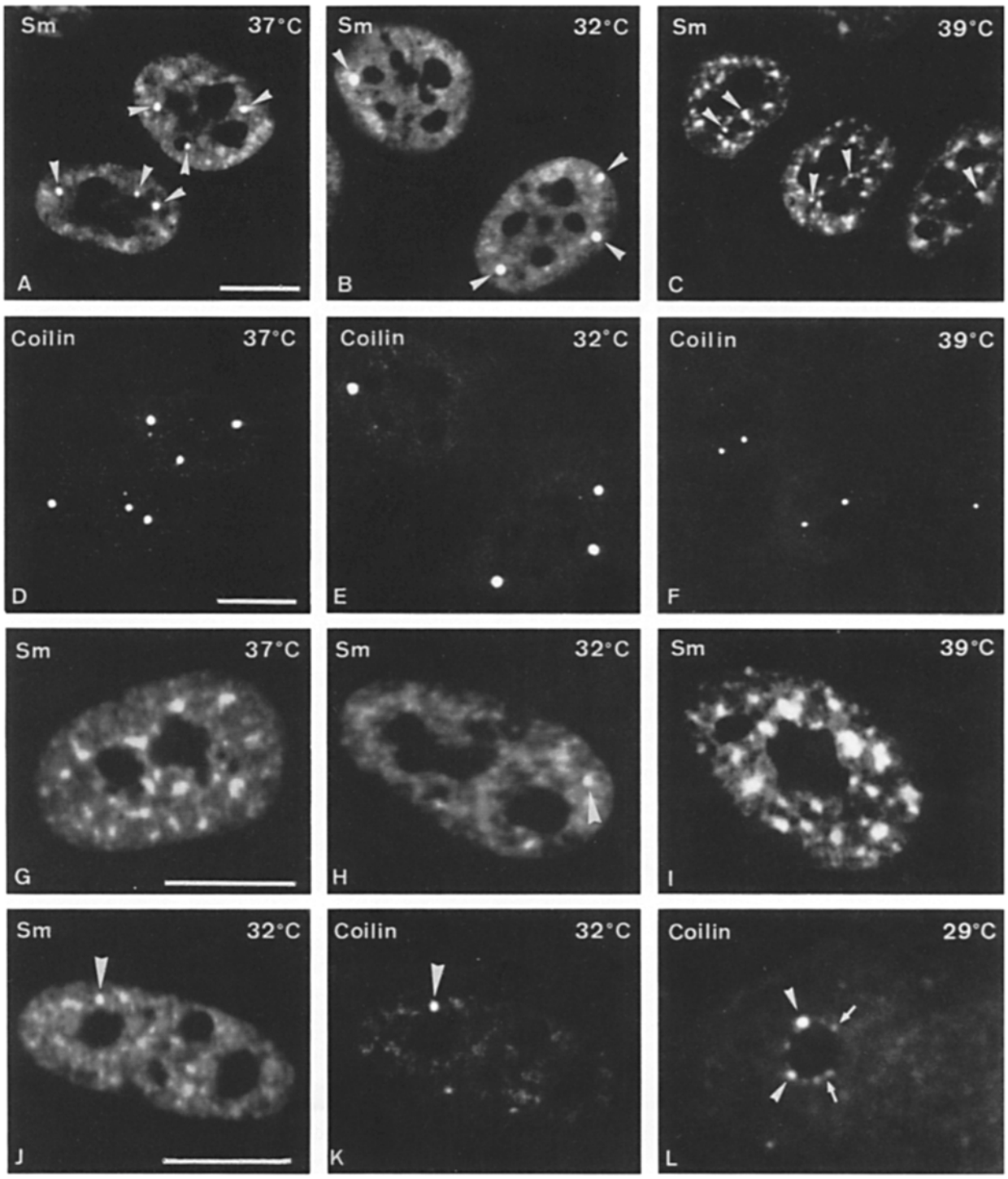

Figure 1. CB size and nuclear snRNP distributions are temperature dependent. (*A–F*) HeLa cells double labeled with an anti-snRNP monoclonal antibody (*A–C*) and rabbit anticoilin antibody (*D–F*). HeLa cells were labeled after 24 h at 37°C (*A* and *D*), 32°C (*B* and *E*), or 39°C (*C* and *F*). The bright foci of snRNP staining which correspond to CBs are indicated with arrowheads in *A–C*. Both the anticoilin and anti-snRNP antibodies show that the CBs/snRNP foci are increased in size at 32°C and markedly smaller at 39°C. At 39°C the nuclear snRNP staining pattern is also significantly altered, appearing predominantly speckled, and the CBs can hardly be distinguished by the anti-snRNP antibody (*C*). (*G–L*) Human primary fibroblasts (line Wl-38) labeled with an anti-snRNP monoclonal (*G–I*) after 24 h at 37°C (*G*), 32°C (*H*) or 39°C (*I*). A predominantly speckled pattern of snRNP staining is seen at 37°C and 39°C. At 32°C the staining is more widespread through the nucleoplasm and distinct foci are apparent (*H*, *arrowhead*). Double labeling fibroblasts grown at 32°C with anti-snRNP (*J*) and anticoilin (*K*) antibodies shows that the foci are CBs. In some fibroblasts grown at 29°C, anticoilin antibodies stain caps around the nucleolus (*L*, *arrows*) as well as prominent CBs (*L*, *arrowheads*). Bar, 10 μm.

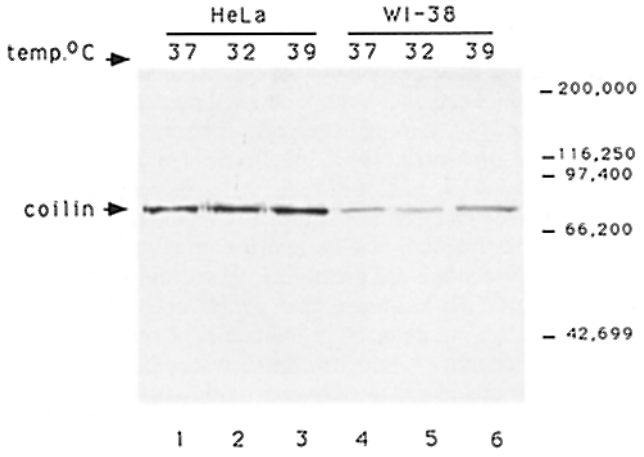

Figure 2. Effect of temperature on p80 coilin levels in HeLa cells and primary fibroblasts. Immunoblot analysis was carried out using a rabbit anticoilin antibody to detect p80 coilin in whole cell extracts prepared from HeLa cells (lanes *1–3*) or primary human fibroblasts (*4–6*) grown for 24 h at either 37°C (lanes *1* and *4*), 32°C (lanes *2* and *5*) or 39°C (lanes *3* and *6*). Equivalent amounts of total protein were loaded in each lane. A single coilin brand migrating with an apparent relative molecular mass of ~80 kD is seen in each case. No significant change in coilin levels is apparent at different temperatures for either cell type. The positions of migration of protein markers of known size are indicated to the right.

(Fig. 1 *L*). This extreme sensitivity of CBs to temperature in primary fibroblasts is not a common feature of primary cells, as primary neurons grown at 37°C show large CBs (cf. Fig. 7). It may instead be connected with the differentiated state of the fibroblast because transformed derivatives of several primary fibroblast lines show prominent CBs at 37°C (unpublished observations).

To extend these observations on temperature-dependent CB formation, western blotting analyses with anticoilin antibodies were carried out on extracts prepared from both HeLa cells and primary human fibroblasts grown at 32, 37, and 39°C respectively (Fig. 2). In both cell types a single coilin band migrating at ~80 kD is observed at each temperature. No increase in the level of p80 coilin was detected at the lower temperatures where CBs are more prominent in situ. Similarly, no decrease was seen at the higher temperatures where CBs are difficult to detect in situ. Thus, similar amounts of p80 coilin are present in cells regardless of whether prominent CBs are detected by immunofluorescence. An important point which emerges from these observations is that the failure to detect CBs in a given cell type grown under specific conditions does not necessarily mean that components of CBs are absent or that CBs cannot rapidly appear. We conclude that both HeLa cells and primary human fibroblasts contain the CB antigen, p80 coilin, which will accumulate under specific conditions in prominent CBs containing splicing snRNPs. CBs therefore appear to be kinetic structures which most likely form as part of, or in conjunction with, a temperature-dependent metabolic process taking place in the nucleus. We propose that this metabolic process is connected with the role of snRNPs in the processing and/or transport of pre-mRNA.

Mitotic Regulation of Coiled Bodies

In addition to the changes observed in interphase nuclei, CBs were also seen to change during the cell division cycle (Fig. 3). Prominent snRNP-containing CBs are observed in interphase nuclei of HeLa cells as illustrated by double labeling with anticoilin antibodies (Fig. 3 *A*) and with a monoclonal antibody specific for splicing snRNPs (Fig. 3 *D*). During mitosis the anticoilin antibodies show a wide-

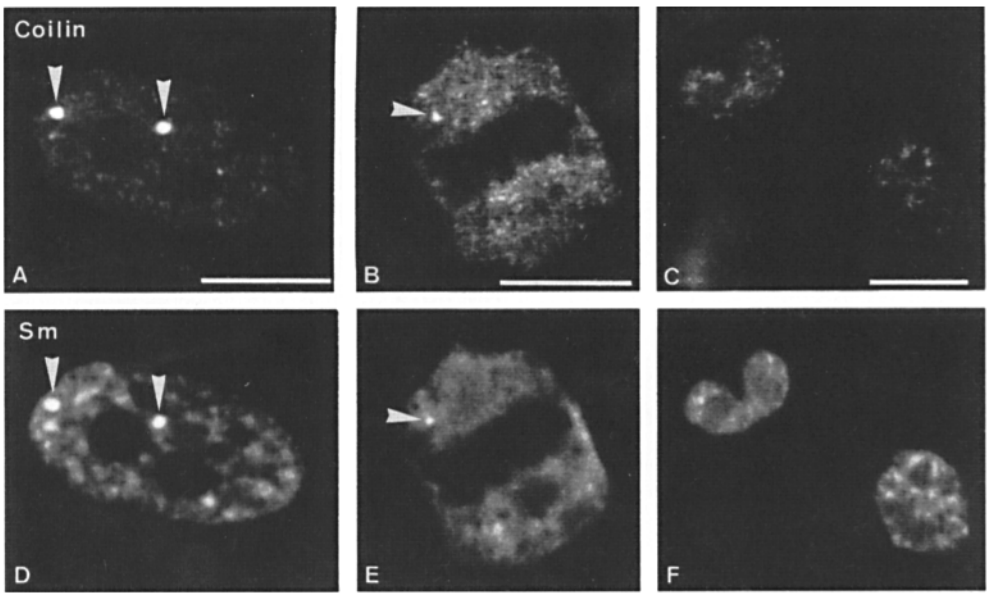

Figure 3. Mitotic regulation of CBs. HeLa cells were fixed with paraformaldehyde, permeabilized with Triton and double labeled with anticoilin (*A*, *B*, *C*) and anti-Sm (*D*, *E*, *F*) antibodies. In interphase cells the anticoilin antibodies reveal prominent coiled bodies (*A*, *arrowheads*). Coiled bodies colocalize with brightly stained foci labeled by the anti-Sm monoclonal antibody (*D*, *arrowheads*). In metaphase cells both anticoilin (*B*) and anti-Sm antibodies (*E*) diffusely stain the cytoplasm and label small CB remnants (*B* and *E*, *arrowheads*). In the nuclei of cells which have just completed mitosis anticoilin antibodies weakly label the nucleoplasm without showing prominent CBs (*C*) and anti-Sm antibodies show strong, widespread nucleoplasmic fluorescence without staining bright foci (*F*). Bar, 10 μm.

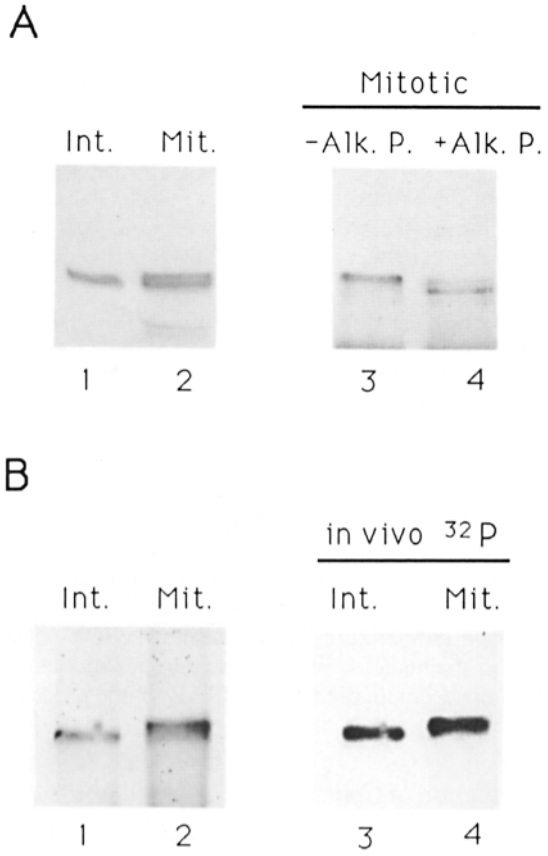

Figure 4. Analysis of p80 coilin in interphase and mitotic HeLa cell extracts. (*A*) Immunoblot analysis of p80 coilin in extracts prepared from HeLa cells in interphase (lane *1*) or HeLa cells enriched for mitotic cells (lane *2*). The mitotic-enriched extract was prepared from cells isolated from plates by mechanical shake off and the interphase extract was prepared from the mitotic-depleted cells left behind on the plate. No drugs were used for these extracts. Equal amounts of protein from each extract were loaded onto the gel as confirmed by Ponceau staining of the nitrocellulose membranes. The additional, slower migrating band detected specifically in the mitotic extract is indicated by an arrowhead. Alkaline phosphatase digestion was performed on p80 coilin immunoprecipitated from extracts prepared from HeLa cells blocked in mitosis by treatment with nocodazole (lanes *3* and *4*). Alkaline phosphatase treatment was done in the presence (lane *3*) or absence (lane *4*) of β-glycerophosphate as inhibitor. Alkaline phosphatase converts the slower migrating, mitotic coilin band (*arrowhead*) to the faster migrating band seen in interphase extracts. (*B*) HeLa cells were arrested in interphase with hydroxyurea (lanes *1* and *3*) and in mitosis with nocodazole (lanes *2* and *4*), labeled in vivo with [^{32}P]orthophosphate and coilin isolated by immunoprecipitation. The immunoprecipitated protein was separated by SDS-PAGE and transferred to a nitrocellulose membrane. Lanes *1* and *2* show immunodetection of coilin using rabbit anticoilin antibodies and lanes *3* and *4* show an autoradiogram of the same blot. Similar results were obtained using interphase and mitotic extracts prepared from HeLa cells that were not blocked with drugs (data not shown).

spread staining with some small remnants of CBs visible (Fig. 3 *B*). In the daughter nuclei of cells which have just completed telophase, anticoilin antibodies give a faint, widespread nucleoplasmic staining with no prominent CBs visible (Fig. 3 *C*). Double labeling with the anti-snRNP monoclonal also shows a widespread labeling in the metaphase cell, but with more concentrated staining in the CB remnant (Fig. 3 *E*; cf. Fig. 3 *B*). It also gives bright, widespread nucleoplasmic fluorescence in the postmitotic cells, without staining foci (Fig. 3 *F*). A more detailed description of p80 coilin and snRNP staining patterns at different stages of mitosis will be presented elsewhere (Ferreira, J., M. Carmo-Fonseca, and A. I. Lamond, manuscript in preparation).

We conclude that CBs are mitotically regulated structures and that both the size of CBs and the amount of splicing snRNPs they contain are greatly decreased during mitosis. The fact that CBs reappear in daughter cells after a lag period, and are not detected in the nuclei of cells that have just completed mitosis, supports the view that CBs are mitotically disassembled and must be reassembled after cell division. The occurrence of a postmitotic lag during which CBs are reassembled may also explain observations that a small fraction of cells in culture appear to lack CBs.

Mitotic Phosphorylation of p80 Coilin

As its staining pattern differs in interphase and mitotic cells, we tested whether coilin is modified during mitosis (Fig. 4). Western blotting analysis was done using whole cell extracts prepared from interphase cells and from cultures enriched for mitotic cells (Fig. 4 *A*). The anticoilin antibody specifically stains a singlet band of ~80 kD in the interphase extract but a doublet in the mitotic extract, with an additional band migrating just above that at ~80 kD (Fig. 4 *A*, lanes *1* and *2*). An extract prepared from cells blocked in metaphase contains predominantly the upper band (Fig. 4 *A*, lane *3*). Alkaline phosphatase converts the upper (mitotic) band to a faster migrating species that approximately comigrates with the ~80 kD coilin band in interphase extracts (Fig. 4 *A*, lanes *3* and *4*). This suggests that p80 coilin is phosphorylated in mitotic extracts. To test directly for coilin phosphorylation, interphase and mitotic extracts were prepared from HeLa cells grown in the presence of [^{32}P]orthophosphate (Fig. 4 *B*). Western blotting analysis with an anticoilin antibody again showed the coilin band from the mitotic extract migrating slightly above the coilin band from the interphase extract (Fig. 4 *B*, lanes *1* and *2*). Autoradiography of this same blot demonstrates that both bands are labeled with [^{32}P]orthophosphate (Fig. 4 *B*, lanes *3* and *4*). We conclude that coilin is a phosphoprotein.

The phosphorylation pattern and phosphoamino acid composition of in vivo ^{32}P-labeled p80 coilin was compared in the interphase and mitotic extracts (Figs. 5 and 6). Tryptic peptides from in vivo labeled coilin were separated in two dimensions and analyzed by autoradiography (Fig. 5). A comparison of the labeled coilin peptides detected in interphase and mitotic extracts demonstrates that: (*a*) in both extracts, p80 coilin is phosphorylated at multiple sites and (*b*) there are at least two additional phosphopeptides in mitotic extracts that are not detected in interphase extracts (Fig. 5 *B, arrowheads*). These two extra peptides were detected specifically in mitotic extracts in several independent in vivo labeling experiments. In parallel, in vivo ^{32}P-labeled p80 coilin was isolated, hydrolysed to amino acids and the phosphoamino acid composition determined by one-dimensional thin layer electrophoresis (Fig. 6). Exclusively phosphoserine residues were detected in coilin isolated from both the interphase and mitotic extracts.

In summary, we conclude that p80 coilin is a phos-

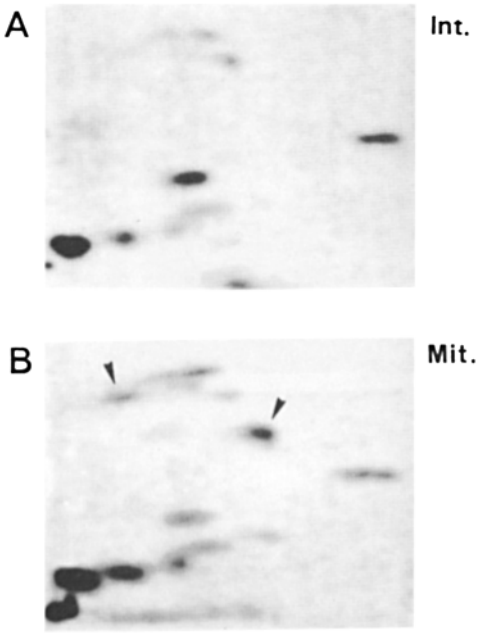

Figure 5. Two-dimensional analysis of tryptic peptides from in vivo labeled coilin. Tryptic peptides of p80 coilin in vivo labeled with [^{32}P]orthophosphate were isolated from extracts of HeLa cells drug-arrested in interphase (*A*) and in mitosis (*B*). The peptides were separated in two dimensions as described in Experimental Procedures and detected using a Phosphorimager (Molecular Dynamics, Inc.) exposed at room temperature for 4 d. The positions of the two peptides specifically detected in mitotic extracts are shown by arrowheads (*B*). Origin is at bottom left hand corner of each plate.

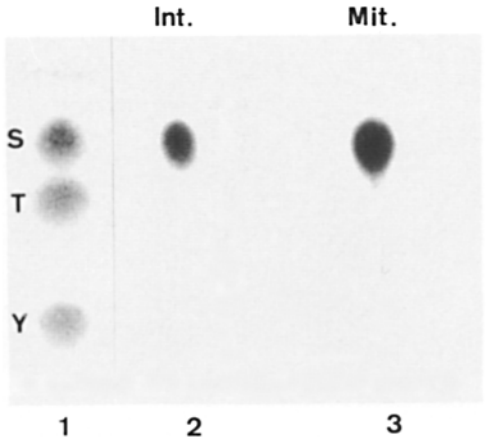

Figure 6. Phosphoamino acid analysis of in vivo labeled coilin. p80 Coilin was in vivo labeled with [^{32}P]orthophosphate, isolated by immunoprecipitation, hydrolyzed to amino acids and analyzed by one-dimensional electrophoresis as described in Materials and Methods. (Lane *1*) Unlabeled phosphoamino acid markers (*S* is phosphoserine, *T* is phosphothreonine, and *Y* is phosphotyrosine) detected by staining with ninhydrin. (Lane *2*) phosphoamino acids detected in coilin isolated from drug-blocked interphase cells (Lane *3*) phosphoamino acids detected in coilin isolated from drug-blocked mitotic cells.

phoprotein which undergoes phosphorylation on at least two additional sites during mitosis. The interphase and mitotic phosphorylation sites are either exclusively, or predominantly, on serine residues. We note that it is not yet possible to unambiguously determine the precise sites of mitotic and interphase phosphorylation as the sequence of the coilin antigen has not been determined.

Detection of Coiled Bodies and p80 Coilin in Nondividing Cells

To extend the analysis of how mitosis affects CBs, mammalian cells that do not undergo mitosis were studied (Fig. 7). Cultures of explanted primary rat neurons were stained with anti-p80 coilin antibodies and viewed by indirect immunofluorescence in the confocal laser scanning microscope (Fig. 7, *A–C*). Large, prominent CBs were observed in the nuclei of all the neuronal cells examined (Fig. 7, *A–C, arrowheads*). These data are consistent with the previous observations of Raska et al. (1990) and support the view that one reason for the apparent absence of CBs from a subpopulation of dividing cells may be the postmitotic lag period during which CBs reassemble. Double labeling of neurons with the anticoilin and anti-snRNP antibodies demonstrated that the neuronal CBs also contain concentrations of splicing snRNPs (Fig. 7, *D–F*, note *yellow* CB in *F*).

Association of p80 Coilin and Coiled Bodies with the Nucleolar Periphery

In addition to staining nucleoplasmic CBs (Fig. 7, *A–C, arrowheads*), the anti-p80 coilin antibodies also labeled "caps" of varying size at the periphery of nucleoli (Fig. 7, *A–C, arrows*). These appear strikingly similar to the "caps" of coilin staining observed in primary human fibroblasts grown at 29°C (cf. Fig. 1 *L*). Nucleolar "caps" were more frequent in older cultures of differentiated neurons (unpublished observations). The larger nucleolar "caps" were also stained by anti-snRNP antibodies (data not shown). As previously reported (Ramon y Cajal, 1903; Hardin et al., 1969), neuronal CBs are often present at the periphery of nucleoli (e.g., CB shown by arrowhead in Fig. 7, *D–F*). These structures were analyzed in more detail by immunoelectron microscopy using anti-coilin antibodies (Fig. 8). The coilin staining is detected by secondary labeling with immunogold, which appears as black grains. Immunolabeling of coilin was always observed on either CBs or specific structures at isolated regions of the nucleolar periphery and was not associated with internal nucleolar structures. The EM pictures of nucleolar-associated and nucleoplasmic CBs show a knotted, fibrillar structure that is heavily labeled with immunogold (Fig. 8, *A* and *B*). Substructure is seen within the CB as regions of higher and lower contrast among the fibers. These pictures are consistent with previous ultrastructural studies of CBs in animal and plant cells (Hardin et al., 1969; Hervas et al., 1980; Moreno Diaz de la Espina et al., 1982; Lafarga et al., 1983; Raska et al., 1990; Schultz, 1990). The anticoilin antibodies also stained structures at the periphery of nucleoli (Fig. 8, *C–F*). In many cases these structures have the appearance of partially assembled CBs which are either continuous with the nucleolus or attached to it by dense fibers, suggesting that CBs may either assemble or disassemble at the periphery of the nucleolus (Fig. 8, *D–F, arrows*). Less pronounced "caps" of coilin staining were also seen (Fig. 8 *C*). It is likely that these EM pictures of perinucleolar coilin labeling correspond to the "caps" detected by immunofluorescence in Fig. 7 (*A–C*).

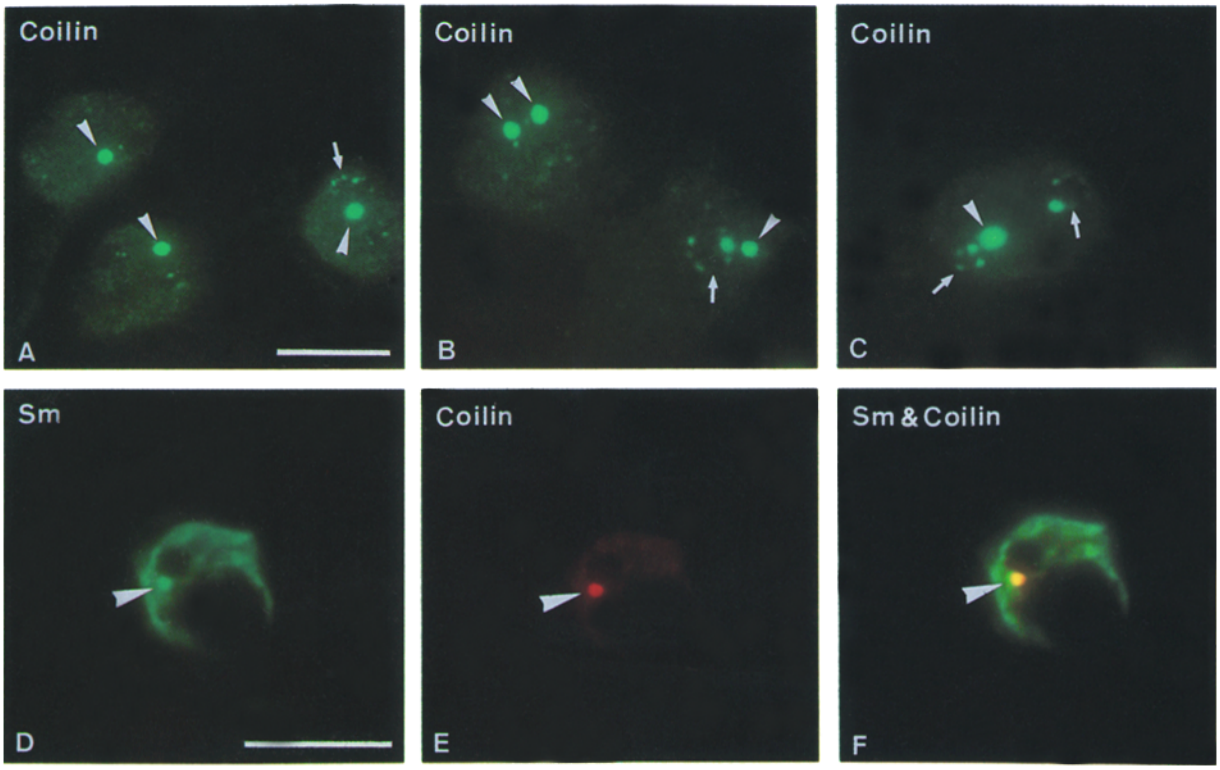

Figure 7. Detection of CBs in differentiated rat hippocampal neurons. Rat hippocampal neurons were maintained in primary culture for 14–17 d and then analyzed by indirect immunofluorescence. Anticoilin antibodies label prominent nuclear CBs in all cells (*A–C, arrowheads*) and also stain "caps" at the periphery of the nucleolus (*A–C, arrows*). Double labeling with an anti-snRNP monoclonal antibody (*D*) and anticoilin antibody (*E*) shows that CBs also contain high concentrations of splicing snRNPs in primary neurons (*D–F, arrowhead*, note *yellow* CB in overlay *F* due to overlap of green and red staining). As well as prominently staining CBs, the anti-snRNP antibody also shows strong, widespread nucleoplasmic labeling in neurons, including local concentrations in some speckled structures. Bar, 10 μm.

Coiled Bodies Contain Fibrillarin but not rRNA or U3 snoRNA

As CBs can clearly interact with the periphery of the nucleolus, we used confocal laser scanning microscopy to test whether they also contain nucleolar components (Fig. 9). In agreement with Raska et al. (1990), we observe that the nucleolar protein fibrillarin and p80 coilin colocalize in CBs (Fig. 9, *A–C*). Colocalization of fibrillarin and coilin is seen for both nucleolar associated (Fig. 9 *C, arrow*) and free nucleoplasmic CBs (Fig. 9 *C, arrowheads*). Similar results were obtained using monoclonal, antipeptide and autoimmune antifibrillarin antibodies (data not shown), indicating that fibrillarin itself, rather than a related protein, is present in the CB. However, double labeling with anticoilin antibodies and antisense probes specific for either the U3 snoRNA (Fig. 9 *D*), 28S rRNA (Fig. 9 *E*) or 5S rRNA (Fig. 9 *F*) shows that none of these RNAs are present in CBs, although they strongly stain the nucleolus (Fig. 9, *D–F*, note that none of the CBs are yellow in the confocal overlays in contrast with the fibrillarin/coilin overlay in Fig. 9 *C*). These data argue against CBs being involved in rRNA metabolism, as suggested in earlier studies (Hardin et al., 1969; Le Beux, 1971; Raska et al., 1990; Lafarga et al., 1991; reviewed in Brasch and Ochs, 1992).

Discussion

In this study, we have shown that the appearance of prominent, snRNP-containing CBs in the nuclei of mammalian cells is temperature dependent. Both the size of CBs, and the amount of splicing snRNPs they contain, significantly decrease when cells are stressed by growth at 39°C. By contrast, cells growing at lower temperatures (29–32°C) show a pronounced increase in the size of CBs relative to those detected at 37°C. Interestingly, a parallel analysis of the CB antigen, p80 coilin, shows that the levels of this protein are not significantly affected by temperature. Coilin was also detected by protein blotting in all cell types studied, regardless of the number or size of CBs they contain as judged by immunofluorescence. These data indicate that the CB is a kinetic nuclear structure.

The dynamic assembly of CBs in the nucleus invites comparison with kinetic cytoplasmic organelles. For example, the Golgi apparatus is a kinetic structure which functions as a specialized metabolic compartment in the multistep process of intracellular protein sorting and secretion. A number of specific protein modification reactions that are required for accurate protein sorting and secretion take place exclusively in the Golgi. The appearance of the Golgi when viewed by microscopy reflects the steady-state level of metabolic flux into and out of this structure. An analogous, multistep process, or "snRNP cycle," is likely to take place within the nucleus during the processing and transport of pre-mRNA. We propose that the CB may act as a specialized compartment for some stage of this nuclear snRNP cycle. In this view, the CB could be involved in, for example, assembly of multi-snRNP complexes, sorting of mRNA from introns

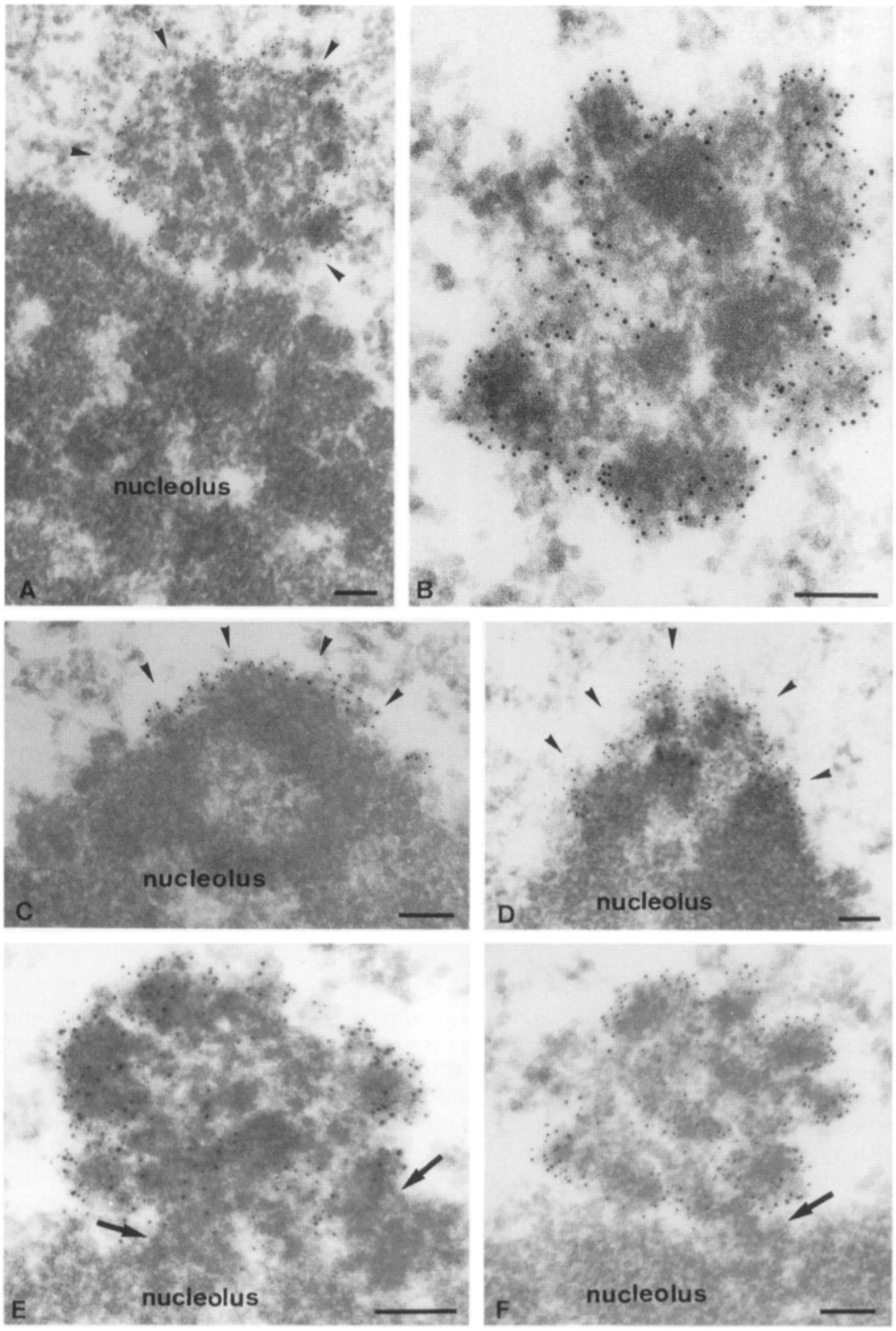

Figure 8. Immunogold labeling of CBs in differentiated rat hippocampal neurons. Immunogold labeling with anticoilin antibodies was done on differentiated rat hippocampal neurons maintained in primary culture for 14–17 d. Discrete CBs are seen in close proximity to the nucleolus (*A*) and independent from the nucleolus (*B*). Coilin antibodies also label a caplike patch at the nucleolar periphery (*C*) and structures resembling partially assembled CBs attached to the nucleolus by continuous dense fibers (*D–F, arrows* point to connecting fibers). Bar, 0.1 μm.

as part of a nuclear export process, recycling of snRNPs from post-splicing complexes, degradation of spliced introns or possibly even act as a site for splicing of specific pre-mRNAs. Each of these activities must occur in the nucleus during mRNA production and could take place in a dedicated compartment. Consistent with this model, the association of splicing snRNPs with CBs has been shown to be transcription dependent (Carmo-Fonseca et al., 1992). The number of CBs per nucleus was also shown to be significantly increased in rat neurons after gene expression was stimulated by osmotic shock (Lafarga et al., 1991), which supports the view that CBs are in some way actively connected with some step in the pathway of gene expression. The main alternative possibility is that CBs are storage compartments where inactive snRNPs accumulate. This seems less likely however because snRNPs are observed to leave CBs when transcription levels fall, at which point the proportion of snRNPs no longer engaged in splicing should increase (Carmo-Fonseca et al., 1992). Contrary to the experimental observations, if CBs were acting as storage sites it would be expected that

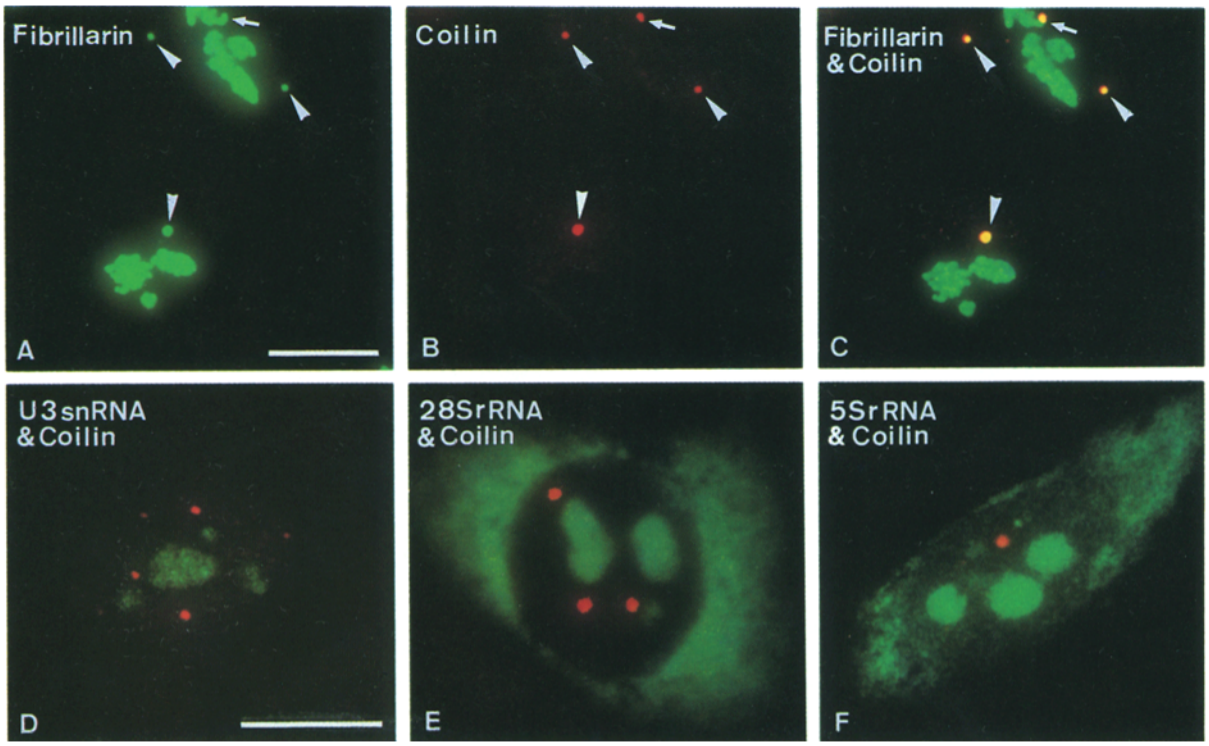

Figure 9. CBs contain fibrillarin but not nucleolar RNAs. (*A–C*) Confocal images of HeLa cells double labeled with an antifibrillarin monoclonal antibody (*A*) and rabbit anticoilin antibody (*B*). The antifibrillarin antibody labels both nucleoli and a few nucleoplasmic foci (*arrowheads in A, green staining*). The extranucleolar foci are also stained by the anticoilin antibody (*B, arrowheads*), as is a nucleolus-associated CB (*B, arrow, red staining*). Precise colocalization of coilin and fibrillarin in CBs is demonstrated when the separate images are superimposed (*C, arrowheads and arrows,* note yellow staining where green and red colocalize). Note that the nucleolus-associated CB (*A–C, arrow*), cannot be distinguished by fibrillarin staining alone (*A*). (*D–F*) Confocal overlays showing the superimposition of separate images of the rabbit anticoilin antibody (red staining) together with in situ labeling of the following nucleolar RNA species (*green staining*); U3 snoRNA (*D*), 28S rRNA (*E*) and 5S rRNA (*F*). Note that in contrast to the confocal overlay of coilin and fibrillarin (*C*), the absence of yellow from D–F shows that none of the CBs are stained by probes for nucleolar RNAs. All three in situ probes specifically label the nucleolus and, as expected, the two rRNA-specific probes also label the cytoplasm. Bar, 10 μm.

the proportion of snRNPs associated with CBs should increase when the levels of pre-mRNA in the nucleus decrease. The data currently available are thus more consistent with CBs playing some form of active role connected with the function of splicing snRNPs. It is important to emphasize, however, that this does not mean that CBs must correspond to sites where splicing of pre-mRNAs takes place. As discussed above, they may be involved in snRNP assembly or disassembly reactions, that occur when snRNPs are not bound to pre-mRNA (or during intron degradation), but are nonetheless essential for the participation of snRNPs in subsequent splicing events. This could explain why the non-snRNP splicing factor SC-35 has not been detected in CBs, although another essential splicing factor, U2AF, is present (Raska et al., 1991; Carmo-Fonseca et al., 1991*b*, 1992; Spector et al., 1991; Huang and Spector, 1992; Zhang et al., 1992). Clearly more work is still required to determine the functional role of the CB organelle.

An important corollary of the CB being a kinetic structure is that its steady-state size will depend upon the metabolic activity of the cell and, like the Golgi, will change according to the ingoing or outcoming flux of components. This means that CBs will only be detected as distinct nuclear structures under certain conditions which will be influenced by the growth and metabolic activity of the cell. Accordingly, we note that the failure to observe prominent CBs by immunofluorescence in certain cell types, or under specific growth conditions, may simply reflect differences in steady-state size of CBs resulting from the relative level, or relative rate, of pre-mRNA synthesis, maturation and transport taking place. It need not imply that the process giving rise to CBs is not taking place or that CBs cannot play an essential role in pre-mRNA maturation in vivo as previously suggested (Huang and Spector, 1992; Spector et al., 1992). Given the high degree of evolutionary conservation of the CB, which is found in plant as well as animal cells, we favor the view that it is likely to perform an important, and possibly essential, function.

Inhibition of transcription causes a major redistribution of p80 coilin and splicing snRNPs within the nucleus (Carmo-Fonseca et al., 1992). In HeLa cells treated with transcription inhibitors, coilin is detected in "caps" at the edge of nucleoli, similar to those shown here in primary fibroblasts and primary neurons which have not been exposed to drugs (Raska et al., 1990; Carmo-Fonseca et al., 1992; cf. this study, Figs 1, 7, and 8). The observed interaction of the CB with the periphery of the nucleolus is also highly consistent with the view that it is a dynamic organelle. These data suggest that the nuclear snRNP cycle, and possibly also CB formation, involve p80 coilin and splicing snRNPs interacting

with the nucleolus, raising a number of interesting possibilities concerning the functional significance of this interaction for mRNA transport or processing.

We show here that CBs contain splicing snRNPs but do not contain 5S or 28S rRNA or the U3 nucleolar snoRNA required for rRNA processing (Fig. 9). CBs also do not contain RNA polymerase I or the nucleolar proteins nucleolin or B23 (Raska et al., 1991). It is likely therefore that the role of the CB is specifically connected with some aspect of the function or assembly/disassembly of nucleoplasmic snRNPs and not with rRNA metabolism as previously suggested (Hardin et al., 1969; Le Beux, 1971; Raska et al., 1990; Lafarga et al., 1991). However, our results confirm that fibrillarin is present in CBs (Raska et al., 1990). Fibrillarin is a highly conserved protein (Jansen et al., 1991), that interacts with multiple nucleolar snoRNPs (Tyc and Steitz, 1989; Baserga et al., 1991). Mutations in the yeast fibrillarin gene impair ribosome production and simultaneously inhibit many steps in the rRNA processing and modification pathway (Tollervey et al., 1991). Intriguingly, these fibrillarin mutations did not prevent the nucleolar accumulation of all seven snoRNAs tested, including U3, but did affect the levels of splicing snRNAs in yeast cells (Tollervey et al., 1991). An interesting possibility, therefore, is that fibrillarin plays a related role in both the nucleolus and CB, but interacts with snoRNPs in the nucleolus and with splicing snRNPs in the CB.

Mitotic phosphorylation is known to play a major role in controlling the disassembly of complex nuclear structures during cell division. For example, disassembly of the nuclear lamina is promoted by M-phase phosphorylation of lamins (reviewed by McKeon, 1991). Lamins are phosphoproteins containing phosphoserine residues (Dessev et al., 1988), certain of which are specifically phosphorylated during mitosis (Ward and Kirschner, 1990). Lamins are also targets for the mitotic kinase $p34^{cdc2}$ (Nakagawa et al., 1990). In this study we provide evidence that a prominent nuclear structure containing splicing snRNPs, i.e., the CB, may also be regulated during mitosis by a similar phosphorylation mechanism. It will now be important to map the precise sites on p80 coilin which are phosphorylated during mitosis and to identify the mitotic kinase(s) responsible for phosphorylating these sites. This requires the sequence of the p80 coilin gene and/or protein to be determined. At present only the sequence of a partial cDNA clone for p80 coilin has been reported, including the 3′ untranslated region of the mRNA and a carboxy terminal coding sequence that could encode ~40 kD of the coilin protein (Andrade et al., 1991). Interestingly, this sequence contains potential targets for the ser/thr-specific $p34^{cdc2}$ mitotic kinase. Coilin has the sequences (lys arg lys *ser* pro lys lys lys) and (lys *ser* pro lys), where the consensus $p34^{cdc2}$ kinase target site is *ser/thr* pro flanked by basic residues, with phosphorylation taking place on the underlined serine or threonine residue (reviewed by Nurse, 1990). Coilin could thus be a direct target for either the mammalian $p34^{cdc2}$ kinase, or another mitotic kinase of similar target specificity. Work is currently in progress to isolate full-length p80 coilin cDNA clones to allow these questions to be answered.

Although transcription is known to be shut down in mammalian cells during mitosis, it isn't known whether pre-mRNA splicing can still take place. It seems probable, however, that splicing is also shut down when cells undergo division. A likely mechanism for inhibiting splicing during mitosis would be for components of the splicing machinery, and/or nuclear structures required for splicing activity, to be inactivated through phosphorylation by mitotic kinases. The mitotic specific phosphorylation of p80 coilin provides a clear demonstration of a protein connected with splicing snRNPs that is specifically phosphorylated during mitosis. It will be interesting now to see whether coilin phosphorylation influences snRNP function in mitotic cells. We therefore anticipate that further structural and functional analyses of the CB may reveal important new insights into the cell biology of gene expression.

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