Molecular Biology of the Cell
Vol. 7, 1137–1151, July 1996

The Dynamics of Coiled Bodies in the Nucleus of Adenovirus-infected Cells

Luisa Rebelo,* Fátima Almeida,* Carlos Ramos,* Kerstin Bohmann,† Angus I. Lamond,† and Maria Carmo-Fonseca*‡

*Institute of Histology and Embryology, Faculty of Medicine, University of Lisbon, 1699 Lisboa Codex, Portugal; and †European Molecular Biology Laboratory, Heidelberg D-69012, Germany

Submitted February 9, 1996; Accepted April 23, 1996
Monitoring Editor: Joseph Gall

The coiled body is a specific intranuclear structure of unknown function that is enriched in splicing small nuclear ribonucleoproteins (snRNPs). Because adenoviruses make use of the host cell-splicing machinery and subvert the normal subnuclear organization, we initially decided to investigate the effect of adenovirus infection on the coiled body. The results indicate that adenovirus infection induces the disassembly of coiled bodies and that this effect is probably secondary to the block of host protein synthesis induced by the virus. Furthermore, coiled bodies are shown to be very labile structures, with a half-life of ~2 h after treatment of HeLa cells with protein synthesis inhibitors. After blocking of protein synthesis, p80 coilin was detected in numerous microfoci that do not concentrate snRNP. These structures may represent precursor forms of the coiled body, which goes through a rapid cycle of assembly/disassembly in the nucleus and requires ongoing protein synthesis to reassemble.

INTRODUCTION

Since the pioneering electron microscopic studies of Bernhard and coworkers, it has became clear that the nucleus of higher eukaryotic cells is a very complex compartment organized into distinct structural domains (Monneron and Bernhard, 1969; Bouteille *et al.*, 1974). Early work from these groups has established clearly that RNA/protein complexes in the nucleus are localized to distinct types of structures that include the nucleolus, the perichromatin fibrils and perichromatin granules, the clusters of interchromatin granules, and the coiled body (reviewed in Monneron and Bernhard, 1969; Smetana and Busch, 1974). Electron microscopic autoradiography has demonstrated further that extranucleolar RNA synthesis occurs in association with perichromatin fibrils (recently reviewed in Fakan, 1994), whereas the small nuclear ribonucleoproteins (snRNPs) involved in splicing of pre-mRNA and several protein splicing factors have been localized in association with perichromatin fibrils, clusters of interchromatin granules, and coiled bodies (reviewed in Spector, 1993). According to the view that splicing occurs cotranscriptionally (Beyer and Osheim, 1988; LeMaire and Thummel, 1990; Bauren and Wieslander, 1994), perichromatin fibrils are likely to represent nascent transcripts with associated snRNPs engaged in active splicing of the newly synthesized pre-mRNA (Fakan, 1994). However, the additional presence of snRNPs in interchromatin granules and coiled bodies remains intriguing and poorly understood.

The coiled body is a distinct subnuclear organelle that contains splicing snRNPs and is intimately associated with the nucleolus (recently reviewed in Bohmann *et al.*, 1995b). Coiled bodies have been observed in almost all cell types examined, ranging from mammals to plants. A specific component of the human coiled body is a novel protein with an M_r of $\sim 80 \times 10^3$ called p80 coilin (Andrade *et al.*, 1991); more recently, a homolog protein named SPH-1 was identified in *Xenopus laevis* (Tuma *et al.*, 1993). In the nucleus of amphibian oocytes, SPH-1 is localized in prominent "spheres" that also contain snRNPs and, therefore, may be closely related or equivalent to coiled bodies (reviewed in Gall *et al.*, 1995; Roth, 1995).

In this work we sought to investigate whether adenovirus infection affects the coiled body. Adenovi-

‡ Corresponding author.

ruses have long been considered an invaluable tool for understanding the mechanisms of pre-mRNA processing in mammalian cells because the maturation of viral RNA is carried out by the host cellular machinery and involves capping, polyadenylation, methylation, and splicing (reviewed in Ziff, 1980; Sharp, 1984). Consistent with these data, we and others have demonstrated previously that the virus subverts the normal subnuclear organization by recruiting the host-splicing snRNPs to the sites of viral transcription (Bridge *et al.*, 1993; Jiménez-Garcia and Spector, 1993; Pombo *et al.*, 1994; Puvion-Dutilleul *et al.*, 1994).

The human adenovirus type 2 (Ad2) causes a productive infection of HeLa cells that proceeds through an infectious cycle of ~36 h (reviewed in Tooze, 1980). This cycle is conventionally divided into early and late stages, separated by the onset of viral replication that occurs ~8 h after infection. During the early phase of infection, ~40% of the viral genome is expressed, encoding a small number of proteins that prime the infected cell for viral DNA replication (Horwitz, 1990). After the onset of viral DNA replication, ~90% of the genome is expressed, and the amount of created RNA increases up to 10-fold. During this phase a single major late promoter (MLP) is strongly activated and encodes a large primary transcript that gives rise to five families of mRNAs (L1–L5) by differential splicing and polyadenylation (Tooze, 1980; Sharp, 1984). Each of these mRNAs has in common a 5′-noncoding region of 201 nucleotides, called the tripartite leader, and is derived by the splicing of three small introns. Transcription from MLP attains a maximum level 18 h after infection and then remains constant for at least 10 h, yielding large amounts of structural polypeptides that comprise the virion particle or are involved in packaging viral genomic DNA (Horwitz, 1990). During this period the viruses compete with the host cell for control of the translational apparatus, and Ad2 has evolved multiple mechanisms that suppress cellular protein synthesis and enhance translation of late viral mRNAs (reviewed in Zhang and Schneider, 1993).

In the present study we report that coiled bodies disassemble in the nucleus of adenovirus-infected cells, and we argue that the effect of adenoviral infection on the coiled body may be secondary to the block of host protein synthesis induced by the virus.

MATERIALS AND METHODS

Cell Culture

HeLa-cell monolayer cultures were maintained in Dulbecco's modified minimum essential medium supplemented with 10% fetal calf serum. Subconfluent cells were infected with wild-type Ad2 at a multiplicity of infection of 20-focus-forming units (ffu) per cell. Virus titers, expressed as ffu/ml, were determined on HeLa monolayers, as described previously (Philipson, 1961). The cells were inoculated with virus in serum-free medium; after incubation for 1–2 h, the medium was removed and replaced by fresh medium supplemented with 10% serum. The virus added was sufficient to infect >80% of cells in all experiments.

For drug experiments fresh culture medium was added and allowed to equilibrate for 1 h. The cells were then exposed to 100 μM anisomycin, 100 μM cycloheximide, or 1 μM emetine (Grollman, 1968; Vazquez, 1979). Stock solutions were prepared in 10% ethanol and stored at −20°C (10 mg/ml anisomycin, 10 mg/ml cycloheximide, and 1 mg/ml emetine). Control experiments were performed in the presence of ethanol (0.1% final concentration).

Antibodies

Monoclonal antibodies were raised against recombinant human coilin by using standard procedures (Harlow and Lane, 1988). Spleen cells from an immunized BALB/c mouse were fused with mouse Ag8 plasmacytoma cells, and the resulting hybridomas were screened by immunofluorescence. The cell line 1D4-δ, which secretes antibodies of the IgG1 class, was derived from a positive clone by limiting dilution in microtiter plates. This hybridoma cell line was then used to induce a peritoneal tumor in pristane-primed adult female mice, and ascitic fluid was collected.

In addition, a rabbit polyclonal serum raised against a His_6–coilin fusion protein (see reference 204 in Bohmann *et al.*, 1995a) was used. Fibrillarin was detected with the monoclonal antibody 72B9 (Reimer *et al.*, 1987). Splicing snRNPs were labeled with monoclonal antibodies Y-12 (Lerner *et al.*, 1981) and 7.13 (Billings *et al.*, 1982) and human autoimmune serum C45 (kindly provided by Dr. W. van-Venrooij).

Probes

Biotinylated 2′-*O*-methyl oligonucleotides were synthesized as described by Sproat *et al.* (1989). Biotin residues (either 2 or 4) were linked at the 5′ terminus via flexible alkyl spacer arms to additional, nonbase-pairing deoxycytidine residues. The exon probe (5′-AIAIUACUIIAAAIACCI-3′) is complementary to the second leader of the major late transcription unit (nucleotides 7, 226-7, 243; Roberts *et al.*, 1986), and the intron probe (5′-IACCAIAUIIACICIICC-3′) is complementary to the first intron of the tripartite leader (nucleotides 6, 189-6, 206; Roberts *et al.*, 1986).

Immunofluorescence and In Situ Hybridization

Cells were grown on 10× 10 mm^2 glass coverslips, rinsed twice in phosphate-buffered saline (PBS), fixed with 3.7% paraformaldehyde in PBS for 10 min at room temperature, and permeabilized with 0.5% Triton X-100 in PBS for 15 min. Alternatively, the cells were rinsed in PBS, permeabilized with 0.5% Triton X-100 in CSK buffer (Fey *et al.*, 1986) for 30 s on ice, and then fixed with 3.7% paraformaldehyde in CSK for 10 min at room temperature.

For immunofluorescence, the cells were washed in PBS containing 0.05% Tween 20 and 0.05% NaN_3 (PBS-Tw) and incubated with primary antibodies diluted in PBS-Tw for 1 h at room temperature. For double-labeling experiments, both primary antibodies were incubated simultaneously for 2 h at room temperature. Antibody-binding sites were detected with secondary antibodies conjugated to either fluorescein isothiocyanate (FITC) or Texas Red (TxRed) (Dianova, Germany; and Vector Laboratories, UK). The samples were mounted in VectaShield (Vector) and sealed with nail polish. Controls were performed using nonimmune sera and a variety of rabbit, human, and mouse antibodies with distinct specificities.

For in situ hybridization, the cells were fixed in paraformaldehyde and permeabilized with 0.2% SDS in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 150 mM NaCl for 5 min with gentle shaking. The cells were then equilibrated in 6× SSPE containing 0.01% Tween 20, treated with 0.5 mg/ml *Escherichia coli* tRNA in 6× SSPE and 5× Denhardt's solution for 30 min, and then incubated with the biotinylated oligonucleotide probes diluted to 2 pM/μl in the same

buffer for 1 h. Alternatively, the samples were denatured in 70% formamide, 2× SSC, and 50 mM sodium phosphate for 3 min at 73°C before hybridization. Hybridization was performed at room temperature in a moist chamber. After hybridization, the cells were washed three times in 6× SSPE at room temperature and twice in 4× SSC and 0.1% Tween 20 at 37°C for 5 min. The cells were then sequentially incubated with FITC-conjugated avidin (5 μg/ml), biotinylated anti-avidin (5 μg/ml), and FITC-avidin (Vector).

Nuclease digestion was performed on cells fixed in paraformaldehyde and permeabilized with SDS before hybridization. The samples were washed twice with 10 mM Tris-HCl, pH 7.5, and incubated with 200 μg/ml heat-inactivated RNase A (Sigma, St. Louis, MO) in the same buffer for 1 h at 37°C.

SDS-PAGE and Immunoblotting

Cells were harvested by scraping with a rubber policeman and lysed by boiling 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 on 8% acrylamide gels and transferred to nitrocellulose membranes. 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 antibody conjugated to alkaline phosphatase (Bio-Rad, Richmond, CA).

Statistical Analysis

The number of coiled bodies per nucleus (N_{CB}) was counted in ~400 cells from each experimental group by using a random sampling procedure. Postmitotic daughter cells were not scored because coiled bodies are not yet formed at this stage (Ferreira *et al.*, 1994). For each group, N_{CB} frequency tables were calculated, and the Kruskal–Wallis (KW) test was applied to investigate the hypothesis that all of the groups had similar distributions (Siegel and Castellan, 1988). The contingency table was used to determine a KW statistic, and the level of significance chosen was $\alpha = 5\%$. The hypothesis that all samples were taken from the same population was rejected if the calculated p-value associated with the KW statistic was <0.05. When the null hypothesis was rejected, the nonparametric test described by Conover (1980) was used to determine which pairs of groups tended to differ; this was done at the same level of significance ($\alpha = 5\%$). The hypothesis that any two groups were taken from the same population was rejected if the calculated p-value was <0.05.

Fluorescence Microscopy

Samples were examined with a Zeiss LSM 310 microscope (Oberkochen, Germany). Confocal microscopy was performed with argon ion (488 nm) and HeNe (543 nm) lasers to excite FITC and TxRed fluorescence, respectively. For double-labeling experiments, images from the same confocal plane were sequentially recorded and superimposed. Alternatively, images were digitized with a Hamamatsu SIT-camera and an ARGUS 10 image processor (Hamamatsu Photonics, Japan). The images were captured with the use of separate filters and then superimposed. To obtain a precise alignment of superimposed images, the equipment was calibrated by using multicolor fluorescent beads (Molecular Probes, Eugene, OR) and a dual-band filter that allows simultaneous visualization of red and green fluorescence.

Electron Microscopy

Immunoelectron microscopy was performed by using anti-coilin monoclonal antibodies and a pre-embedding technique as described previously (Ferreira *et al.*, 1994). The cells were sequentially incubated with monoclonal antibody 1D4-δ (diluted 1:1000 in 0.1% gelatin, 1% bovine serum albumin, 0.15 M NaCl, and 0.1 M Tris-HCl, pH 7.4) and goat anti-mouse IgG coupled to 5-nm gold particles (Serotec, Oxford, UK; diluted 1:50 in 0.1% gelatin, 1% bovine serum albumin, 0.15 M NaCl, and 0.1 M Tris-HCl, pH 7.4). After immunogold labeling, the cells were post-fixed, dehydrated, and embedded in Epon (Ferreira *et al.*, 1994). Ultrathin sections were observed with a Jeol 100CXII electron microscope operated at 80 kV.

RESULTS

Adenoviral RNA Is Not Detected in Coiled Bodies

HeLa cells were infected with Ad2 and harvested 14–16 h after infection. The cells were fixed with paraformaldehyde, permeabilized with SDS, and incubated with a 2′-*O*-methyl ribonucleotide probe complementary to the second leader of the major late transcription unit (Figure 1). This probe, which hybridizes to unspliced and spliced RNAs, produced an intense labeling of both the nucleus and the cytoplasm from infected cells (Figure 1A), whereas it did not stain uninfected cells (Figure 1C). In infected cells, the staining was completely abolished when digestion with RNase A was performed before hybridization (Figure 1B), indicating that the modified riboprobe is not hybridizing with the viral single-stranded DNA molecules that accumulate in the nucleus. In contrast, when DNA probes are used, it is necessary to digest the samples with RNase and DNase to discriminate between RNA and single-stranded DNA (Jiménez-Garcia and Spector, 1993; Pombo *et al.* 1994; Zhang *et al.*, 1994). Thus, 2′-*O*-alkyloligoribonucleotides are particularly useful as antisense probes to localize Ad2 RNA molecules in situ.

To specifically visualize unspliced viral RNA, we made use of a riboprobe complementary to the first intron of the tripartite leader (Figure 1). Surprisingly, this probe failed to produce any labeling unless the cells were denatured before hybridization. Under these conditions, the probe stained exclusively the nuclei of infected cells (Figure 1, D and F), and digestion with RNAse A confirmed that the riboprobe hybridized specifically to RNA molecules (Figure 1E). When the exon probe was used to perform hybridization under the same denaturation conditions, the results were similar to those obtained in the absence of denaturation (our unpublished observations).

Within the nucleus both the exon and the intron probes revealed a complex pattern of ring-like structures (Figure 1, A and D), similar to that observed previously when in situ hybridization was performed with a genomic Ad2 probe (Pombo *et al.*, 1994). To analyze whether coiled bodies are part of the intranuclear domains containing Ad2 RNA, we performed immunolocalization studies by using antibodies to the coiled-body protein p80 coilin. Two types of anti-coilin antibodies were used: a mouse monoclonal antibody raised against recombinant human coilin (see MATERIALS AND METHODS)

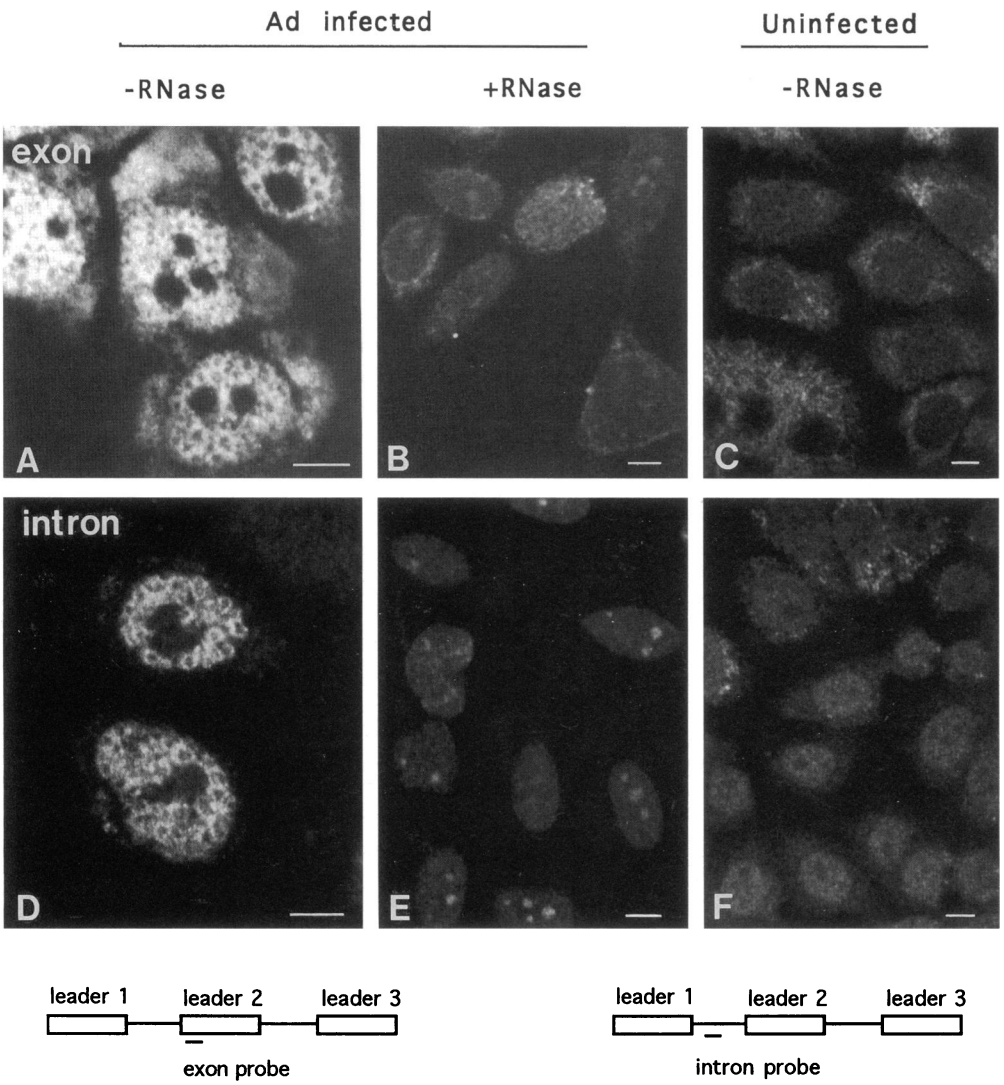

Figure 1. In situ detection of Ad mRNA. HeLa cells were infected for 16 h and hybridized with biotinylated 2′-*O*-methyl oligonucleotides (A and D). The exon probe is complementary to the second leader of the major late transcription unit, and the intron probe is complementary to the first intron of the tripartite leader. In the diagram, boxes represent exons and lines represent introns. As controls, infected cells were digested with RNase A before hybridization (B and E); alternatively, hybridization was performed on noninfected cells (C and F). Bar, 10 μm.

and a rabbit polyclonal serum raised against a His_6–coilin fusion protein (Bohmann *et al.*, 1995a). Western blot analysis of total cellular protein extracts shows that both antibodies react specifically with p80 coilin in either uninfected or Ad2-infected cells. In addition, the data indicate that the amount of p80 coilin per cell remains approximately constant throughout infection (Figure 2). By immunofluorescence, both antibodies revealed the presence of coiled bodies in the nucleus of either uninfected or infected cells. However, most uninfected cells contained one to five coiled bodies per nucleus, whereas infected cells were mostly devoid of coiled bodies or contained only one or two (Table 1). Double-labeling experiments using anti-coilin antibodies, and either the exon or the intron probe failed to detect any accumulation of viral RNA in the coiled body (Figure 3).

Because coiled bodies in noninfected cells are enriched in snRNPs (Lamond and Carmo-Fonseca, 1993) and because snRNPs redistribute in the nucleus of infected cells colocalizing with the sites of viral transcription (Pombo *et al.*, 1994), we sought to determine whether snRNPs persist in the coiled bodies of infected cells. Double-labeling experiments with either the exon probe and anti-Sm antibodies or anti-coilin and anti-Sm antibodies confirm that snRNPs redistribute and colocalize with viral RNA (our unpublished observations) and demonstrate that snRNPs are still present in coiled bodies (Figure 4). However, in contrast with noninfected cells, coiled bodies no longer appear to represent sites of higher snRNP concentration in the nucleus (Figure 4, compare A and B).

In conclusion, the results show that after Ad2 infection coiled bodies become scarcer and do not

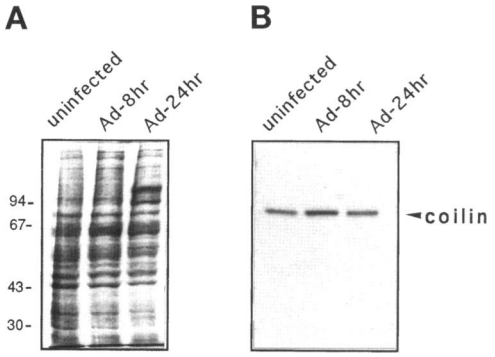

Figure 2. Specificity of anti-coilin antibodies. HeLa cell extracts (~10^5 cells per lane) were prepared from uninfected and Ad-infected cells 8 and 24 h after infection. The proteins were fractionated on 8% SDS-PAGE, transferred to nitrocellulose, and immunoblotted. (A) Coomassie Blue-stained gel. (B) Immunoblot probed with rabbit polyclonal serum 204.3. M_r markers are shown on the left (in kDa).

Table 1. The number of coiled bodies per nucleus (N_{CB}) in uninfected and adenovirus-infected cells

	Uninfected	Infected (16 h p.i.)
N_{CB}	% of cells	% of cells
0	19	79
1	25	17
2	36	4
3	14	0
4	4	0
5	2	0

In each group 300–400 cells were analyzed. Coiled bodies were identified as brightly fluorescent foci with an apparent diameter of ≥0.8 μm. Microfoci (~0.3 μm) labeled by anti-coilin antibodies were not included in the analysis. Ad2 infection was confirmed by in situ hybridization with the exon probe. Statistical analysis indicated that N_{CB} differs between the two groups.

associate with either spliced or unspliced viral mRNA. Therefore, it is unlikely that coiled bodies play a direct role in the processing of adenoviral RNA.

Adenovirus Infection Induces the Disassembly of Coiled Bodies

In the majority of cells harvested 16 h after infection, coiled bodies were no longer visible (Table 1). How-

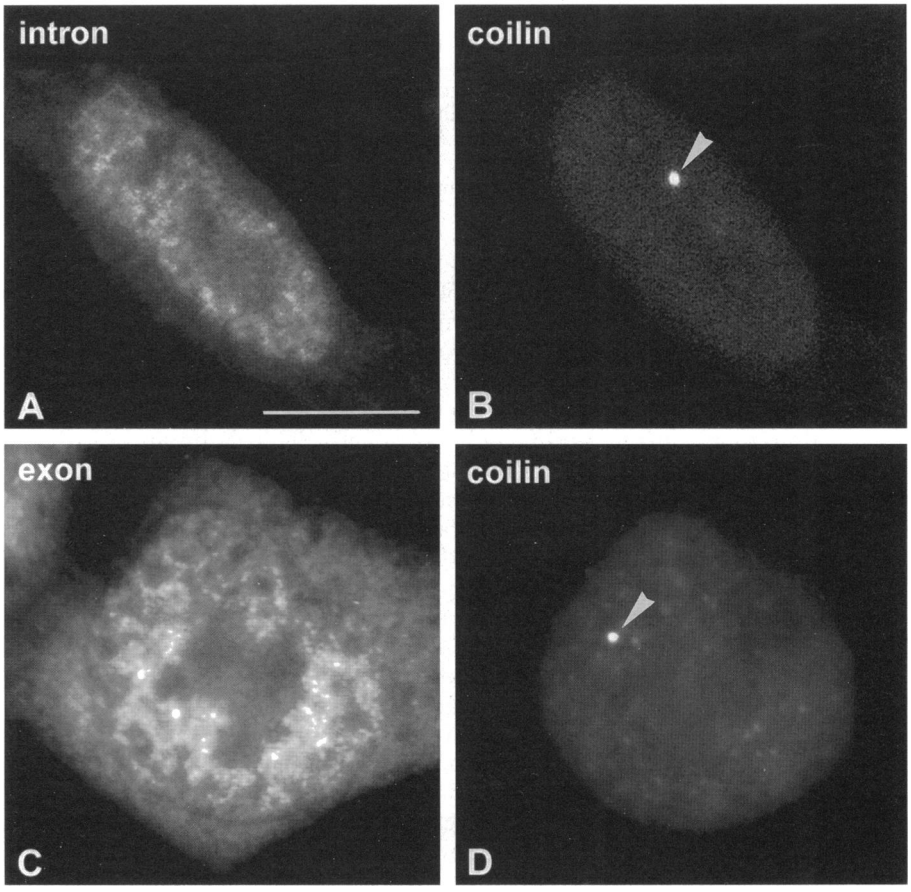

Figure 3. Ad mRNA is not detected in coiled bodies. HeLa cells were infected for 16 h. In situ hybridization with either the intron (A) or the exon (C) probes was combined with immunofluorescence using anti-coilin serum 204.3 (B and D). Arrowheads indicate coiled bodies. Bar, 10 μm.

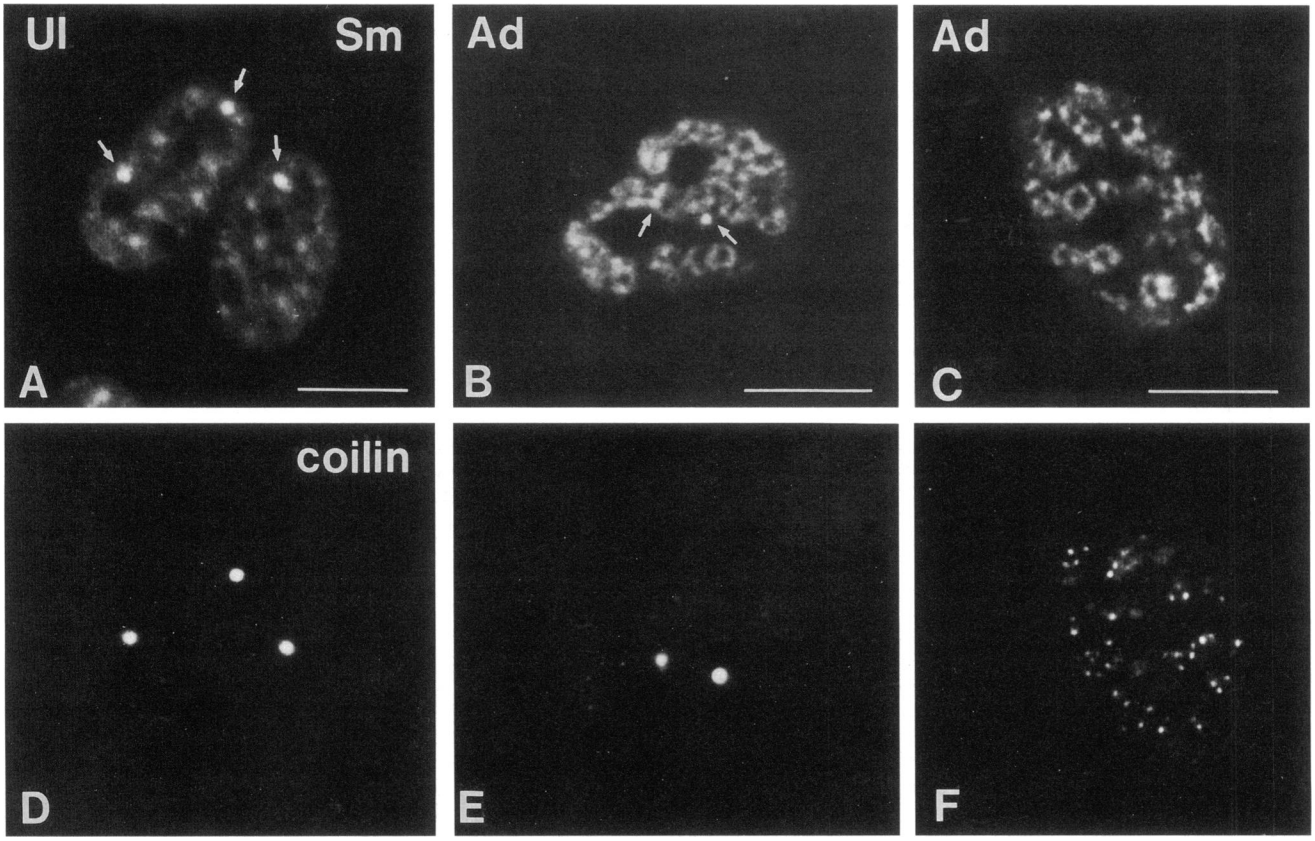

Figure 4. The distribution of snRNPs and coilin in Ad-infected cells. Double-labeling experiments were performed using anti-Sm monoclonal antibody (A–C) and rabbit anti-coilin serum (D–F). In uninfected HeLa cells (A and D), splicing snRNPs are highly concentrated in coiled bodies (arrows). At 16 h after Ad infection, snRNPs are recruited to the sites of viral transcription (B and C); in some cells, coiled bodies are still present (B and E, arrows); however, in the majority of infected cells, coiled bodies have disassembled, and coilin is detected in 50–100 microfoci that do not seem to concentrate snRNPs (C and F). Bar, 10 μm.

ever, both the mouse monoclonal and the rabbit polyclonal anti-coilin antibodies revealed 50–100 microfoci dispersed throughout the nucleoplasm (Figure 4F). In order to further characterize these structures, we performed immuno-electron microscopy using the monoclonal antibody 1D4-δ. Figure 5 depicts a representative staining of control, uninfected HeLa cells. The immuno-gold particles are predominantly associated with coiled bodies, i.e., round structures which have a diameter of 0.5-1 μm and appear to consist of a tangle of loosely coiled threads (Figure 5B, large arrow). In addition, there is a specific labelling of small dense spheres of approximately 0.05 μm in diameter (Figure 5B, arrowheads). Upon adenovirus infection, typical coiled bodies are still visible in cells at early stages of infection (Figure 6, A and B). However, at later stages of infection the gold particles decorate dense, homogeneous structures with a diameter of approximately 0.3 μm (Figure 6C).

Double-labelling experiments using either anti-Sm and anti-coilin antibodies (Figure 4C and F), the intron or exon probes and anti-coilin antibodies, or anti-fibrillarin and anti-coilin antibodies (Figure 7 and unpublished observations) demonstrate that the microfoci contain coilin and fibrillarin but do not concentrate either snRNPs or Ad2 RNA. Thus, we conclude that adenovirus infection induces the formation of intranuclear dense microbodies which contain coilin and fibrillarin but are distinct from coiled bodies.

Coiled Bodies Disassemble in the Presence of Protein Synthesis Inhibitors

Having established that Ad2 infection results in the loss of coiled bodies, we next sought to investigate which mechanism is responsible for this effect. Because the disassembly of the coiled body occurs during the late phase of Ad2 infection and because this stage is marked by the inhibition of cellular protein synthesis (Zhang and Schneider, 1993), we decided to analyze the effects of drugs that block protein synthesis on coilin and the coiled body. Uninfected cells were incubated with anisomycin and harvested at 1-h intervals. For each time point, immunofluorescence was

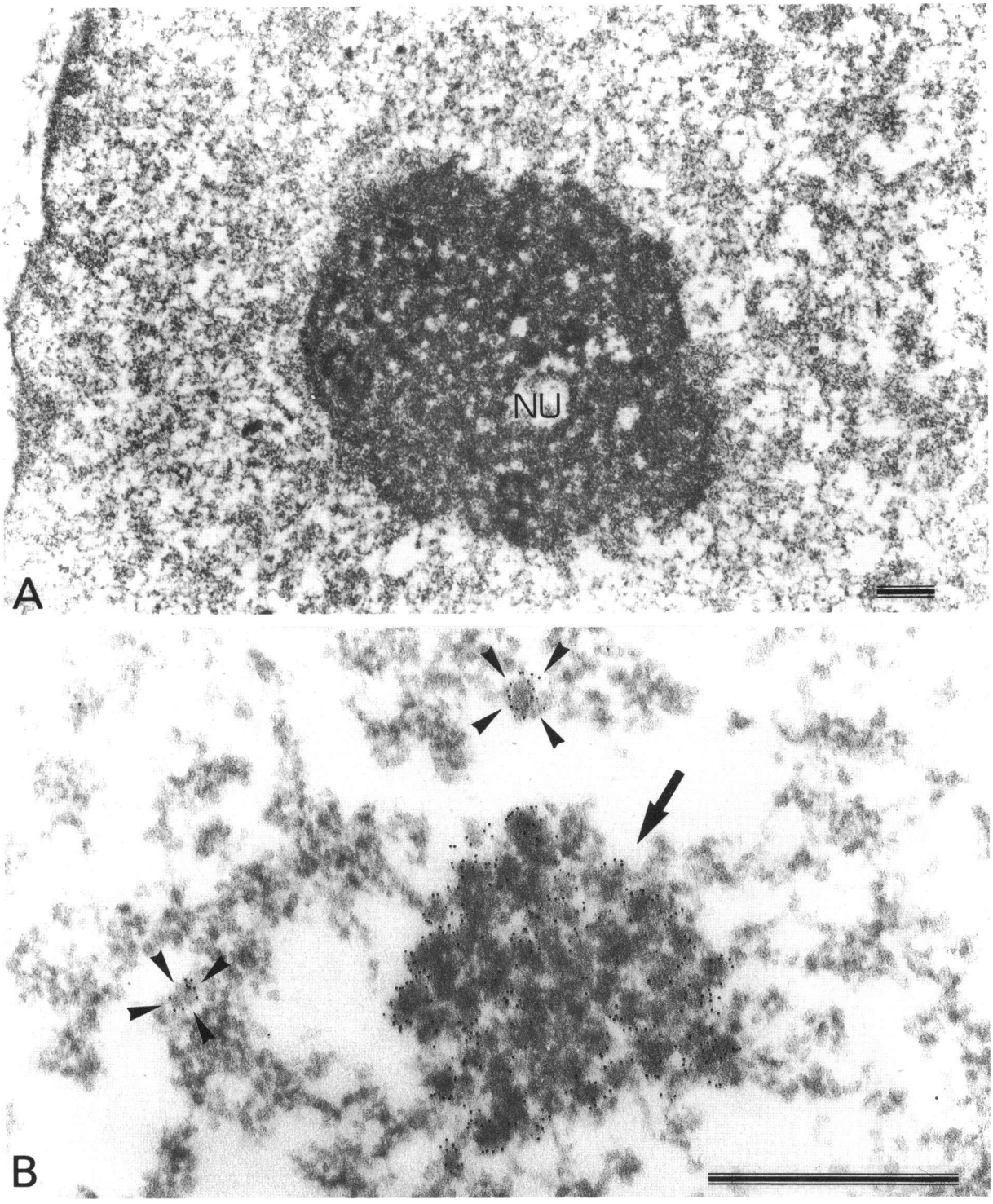

Figure 5. Immunoelectron microscopic localization of coilin in uninfected cells. HeLa cells were immunogold-labeled with the anti-coilin monoclonal antibody 1D4-δ. In these cells, the nucleolus (NU) is typically reticulated, with numerous fibrillar centers. B shows that immunogold particles are specifically localized on coiled bodies that have a diameter of 0.5–1 μm and seem to consist of a loose tangle of coils (large arrow); in addition, there are gold particles associated with microspherules of ~0.05 μm in diameter that appear dispersed in the nucleoplasm (arrowheads). Bar, 0.5 μm.

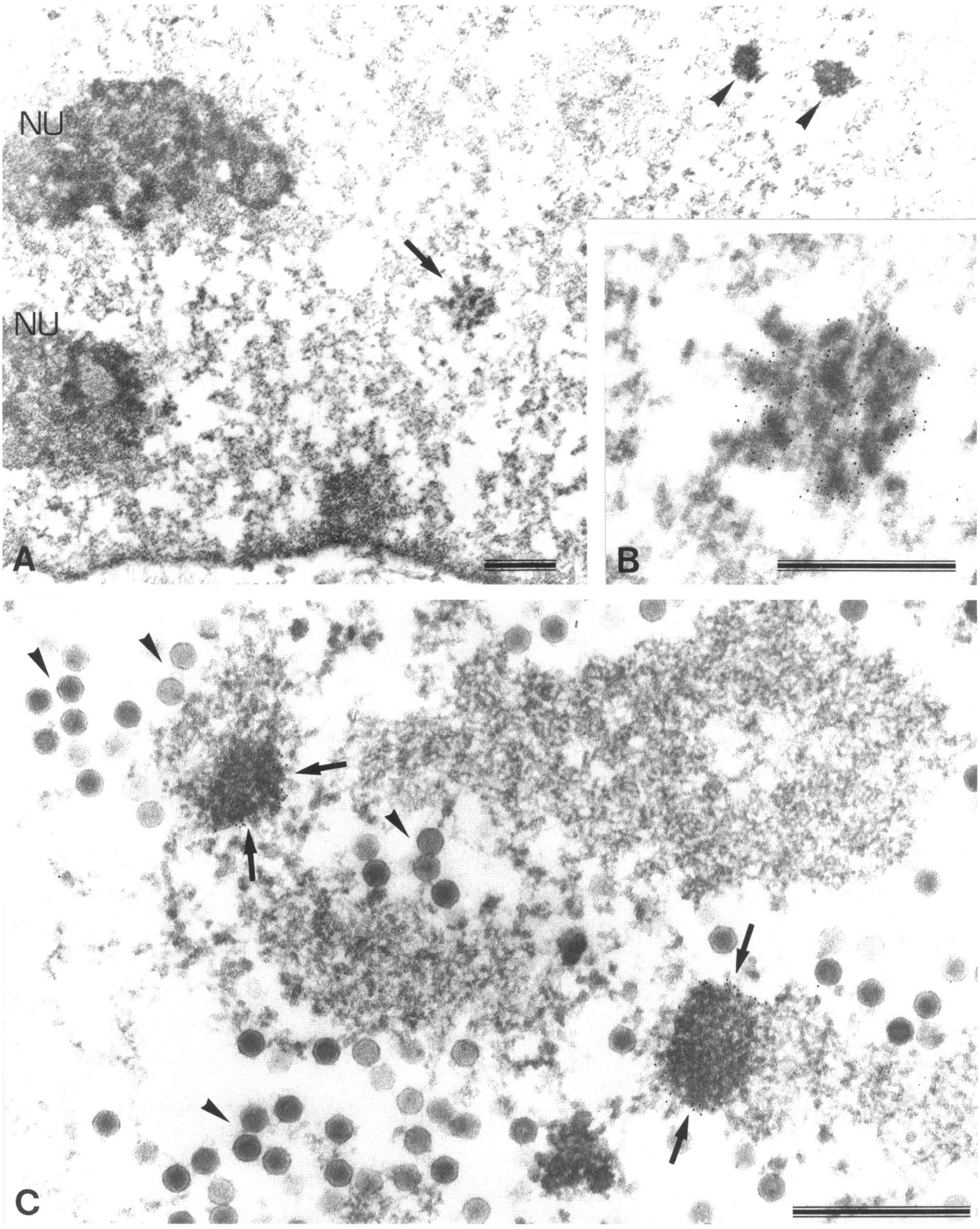

Figure 6. Immunoelectron microscopic localization of coilin in Ad-infected cells. HeLa cells were harvested 16 h after infection and immunogold labeled with the anti-coilin monoclonal antibody 1D4-δ. In some cells, coiled bodies are still visible (A, arrow). Arrowheads in

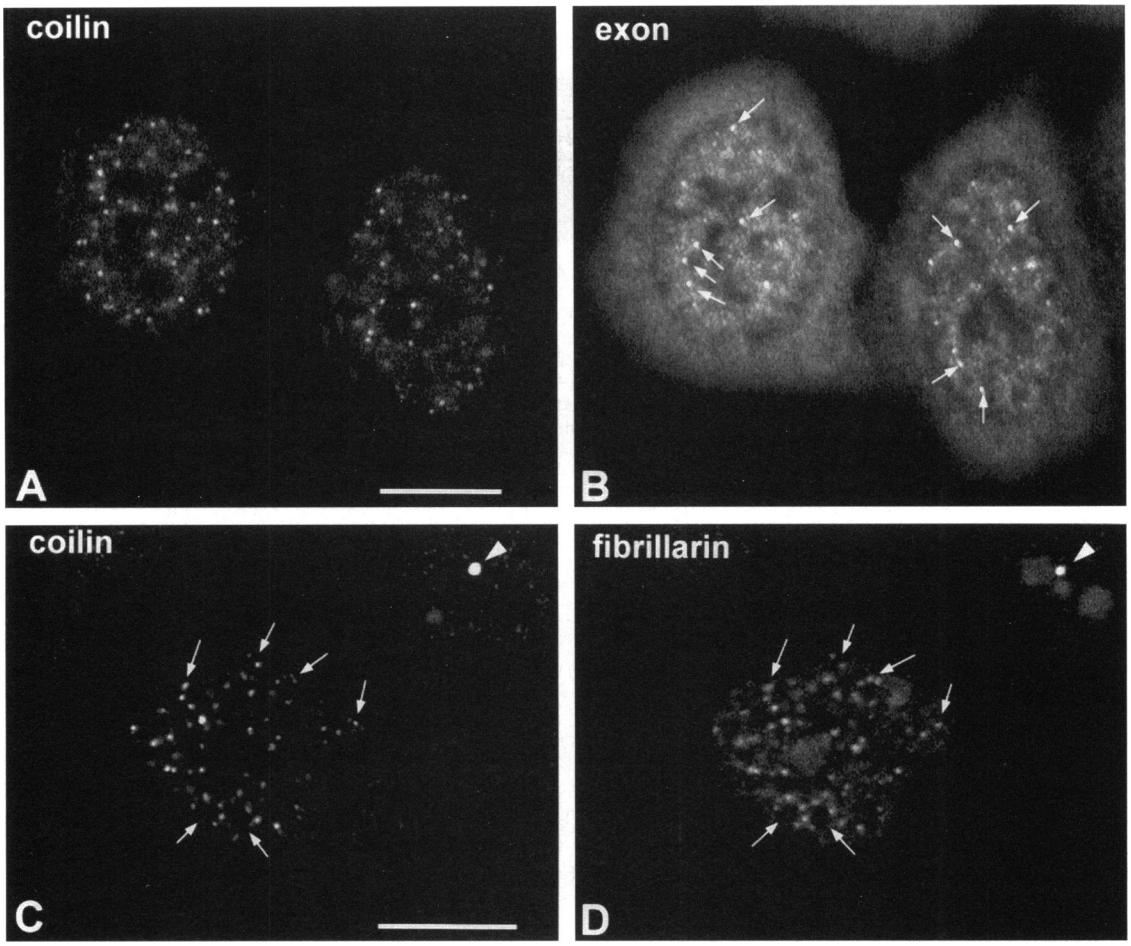

Figure 7. The coilin microfoci induced by Ad contain fibrillarin. Ad2-infected HeLa cells were harvested 16 h after infection and double-labeled using either the exon probe and anti-coilin antibody (A and B) or the anti-fibrillarin and anti-coilin antibodies (C and D). The exon probe produces an intense labeling of dot-like structures (B, arrowheads) that do not colocalize with coilin microfoci (A). In contrast, fibrillarin colocalizes with coilin in the microfoci (C and D, small arrows); on the upper right corner these panels depict an uninfected cell containing a typical coiled body (arrowheads). Bar, 10 μm.

performed with anti-coilin antibodies, and the number of coiled bodies per nucleus was counted in ~400 cells. The results show that anisomycin induces a progressive decrease of coiled bodies in the nucleus. After 2 h of treatment, the average number of coiled bodies per nucleus was reduced by one-half, and after 4–5 h of drug treatment, ~70% of cells were devoid of coiled bodies (Figure 8). In contrast, a parallel Western blot analysis revealed that the amount of p80 coilin per cell was not significantly altered after 5 h of drug treatment (Figure 9D). Interestingly, the nuclei of anisomycin-treated cells often contained coilin microfoci, which appear very similar to those observed in adenovirus-infected cells (Figures 9B and 11, fluorescence microscopy; Figure 10B, electron microscopy). Double-labeling experiments further confirmed that the anisomycin-induced microfoci contain fibrillarin but not snRNPs (Figure 11); they also revealed that in anisomycin-treated cells snRNPs tend to accumulate in enlarged "speckles," which are known to correspond to clusters of interchromatin granules (Figure 11B). After removal of anisomycin from the culture medium, the microfoci disappeared, whereas snRNP-containing coiled bodies reappeared and reached numerical values similar to those observed in nontreated cells (Figures 8 and 9).

Treatment of uninfected HeLa cells with either cycloheximide or emetine for 5 h also induced a decrease of coiled bodies in the nucleus (Table 2) and the ap-

(Figure 6 cont.) A point to virus-induced dense microbodies that are not labeled by anti-coilin antibody. B depicts a large magnification of the coiled body shown in A. At later stages of infection, when viral particles accumulate in the nucleoplasm (arrowheads), the anti-coilin antibody labels homogeneous dense spheroidal structures with ~0.3 μm in diameter (C, arrows). Nu, nucleolus. Bar, 0.5 μm.

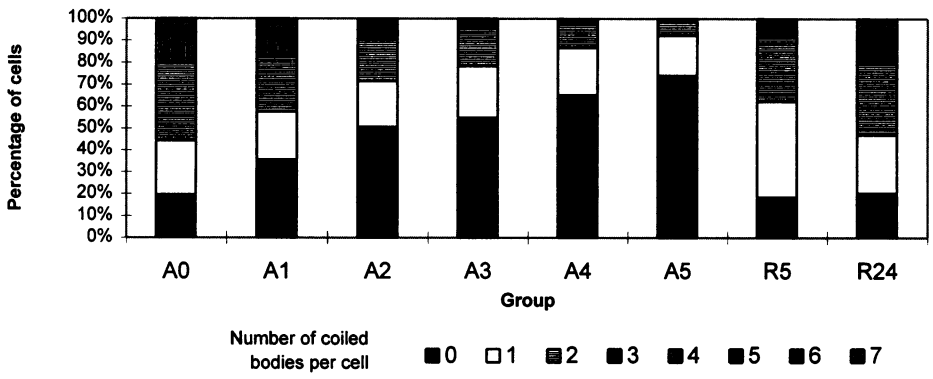

Figure 8. Anisomycin induces a reversible loss of coiled bodies. Quantification of the number of coiled bodies per cell nucleus (N_{CB}) was performed in control cells (A_0 = untreated), in cells treated with anisomycin for 1–5 h (A_1 = anisomycin 1 h; A_2 = anisomycin 2 h; A_3 = anisomycin 3 h; A_4 = anisomycin 4 h; A_5 = anisomycin 5 h), and in cells treated for 5 h and then allowed to recover for 5 or 24 h (R_5 = recovery 5 h; R_{24} = recovery 24 h). At each time point, ~400 cells were randomly selected and analyzed. The figure depicts the calculated frequencies of N_{CB}. Statistical analysis indicated that treatment with anisomycin induced significant changes in coiled-body frequency at all time points examined, whereas after 24 h of recovery the distribution of N_{CB} did not differ from that observed in untreated cells. Similar results were obtained in another two independent experiments.

pearance of coilin microfoci (our unpublished observations).

In summary, the results show that coiled bodies are very dynamic structures that require ongoing protein synthesis and indicate that the effects of adenovirus infection on the coiled body are probably secondary to the block of host protein synthesis induced by the virus.

DISCUSSION

In this study, we show that the coiled body is a very dynamic intranuclear structure that requires ongoing protein synthesis. The observed disappearance of coiled bodies induced by protein synthesis inhibition is consistent with at least two alternative models. 1) Coiled bodies have a high turnover in the nucleus, and inhibition of protein synthesis prevents their reassembly; or 2) coiled bodies disassemble in the presence of protein synthesis inhibitors. Consistent with the first model, Wu and coworkers found that human p80 coilin is rapidly targeted to the amphibian sphere organelles, where it replaces the endogenous SPH-1 protein (Wu *et al.*, 1994). Because the level of p80 coilin remains approximately unchanged after blocking protein synthesis (Figures 2 and 9), we conclude that this protein is not rapidly turning over in the cell. Thus, if the SPH-1 protein can be rapidly replaced by p80 coilin in spheres, it is more likely that these structures go through a rapid cycle of assembly/disassembly in the nucleus.

Our quantitative data indicate that treatment of cultured cells with either anisomycin, cycloheximide, or emetine causes a significant decrease in the number of coiled bodies per nucleus and in the case of anisomycin this effect is fully reversed upon removal of the drug from the culture medium. Furthermore, inhibition of protein synthesis caused by either adenovirus-infection or drugs is shown to induce the appearance of 50–100 microfoci per nucleus, which contain coilin and fibrillarin but do not concentrate snRNPs. At the electron microscopic level the coilin positive microfoci appear as dense aggregates which are clearly distinct from coiled bodies (cf Figures 5 and 6). Possibly, these structures correspond to the dense bodies described by Lafarga *et al.* (1994) in the nucleus of supraoptic neurons from rats injected with cycloheximide. A major conclusion from these results is that coilin may be present in nuclear foci which are not coiled bodies. This emphasizes the importance of electron microscopic studies in the identification and analysis of the coiled body.

Interestingly, the coilin microfoci induced by adenovirus or protein synthesis inhibitors are very similar to the so-called pre-coiled bodies that we have previously observed during early mouse embryogenesis, before the appearance of typical coiled bodies (Ferreira and Carmo-Fonseca, 1995). We propose that in actively growing somatic cells the pre-coiled bodies are rapidly incorporated into coiled bodies and thus are not easily detected by light microscopy. Upon inhibition of protein synthesis, the assembly process may be impaired and consequently the precoiled bodies accumulate and become conspicuous in the nucleus.

The observation that coiled bodies are very prominent when cells are either incubated at low temperatures (Carmo-Fonseca *et al.*, 1993) or taken from hibernating animals (Malatesta *et al.*, 1994) is also consistent with the idea that coiled bodies go through an assembly/disassembly cycle in the nucleus (see Lamond and Carmo-Fonseca, 1993). In this regard it is noteworthy that typical coiled bodies are replaced by "loosened" coiled bodies upon arousal from hibernation (Malatesta *et al.*, 1994). Possibly, at lower temperatures the normal disassembly of coiled bodies is slowed down and therefore the coiled structure appears more perceptible, whereas in the actively me-

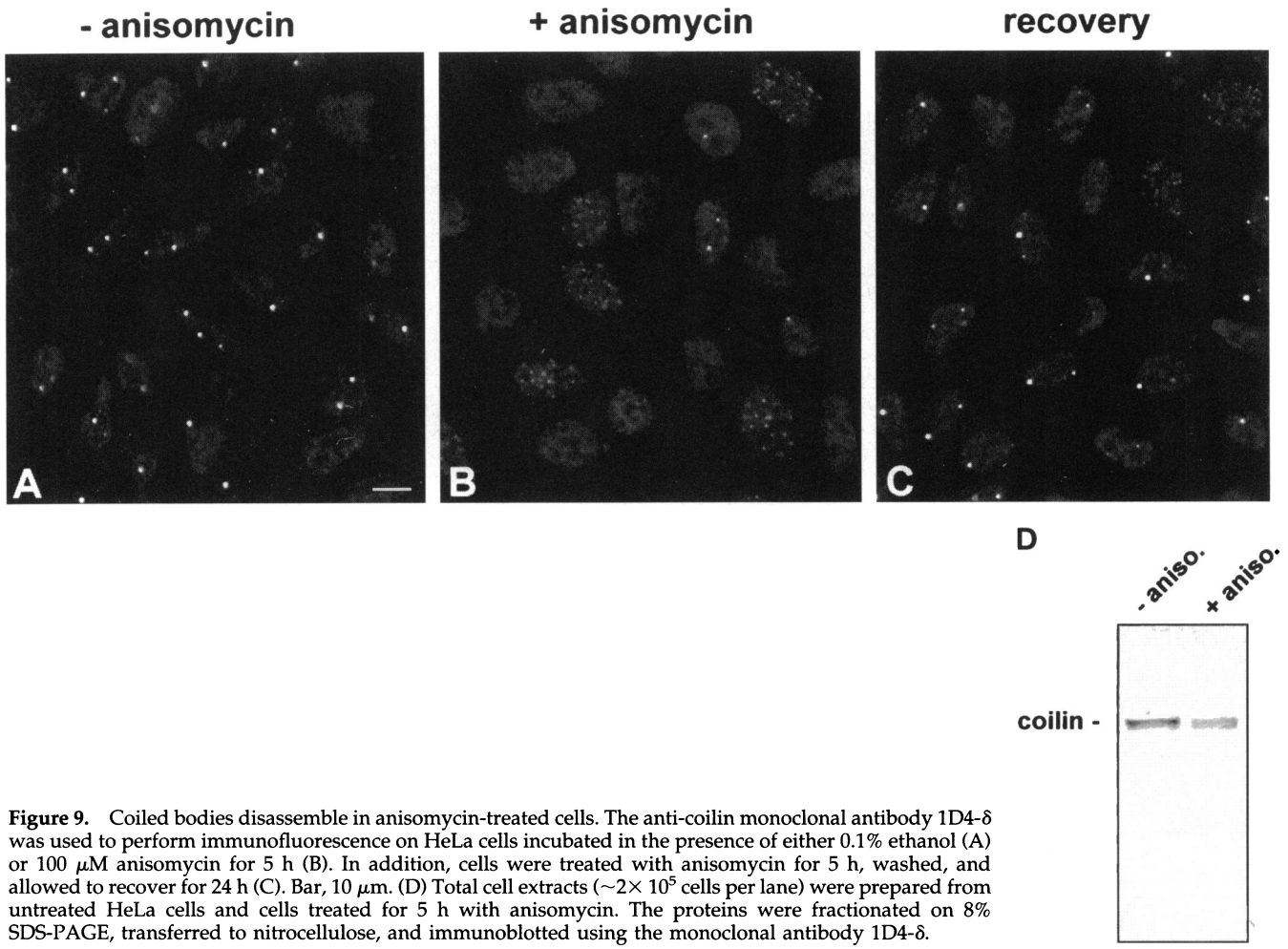

Figure 9. Coiled bodies disassemble in anisomycin-treated cells. The anti-coilin monoclonal antibody 1D4-δ was used to perform immunofluorescence on HeLa cells incubated in the presence of either 0.1% ethanol (A) or 100 μM anisomycin for 5 h (B). In addition, cells were treated with anisomycin for 5 h, washed, and allowed to recover for 24 h (C). Bar, 10 μm. (D) Total cell extracts (~2×10^5 cells per lane) were prepared from untreated HeLa cells and cells treated for 5 h with anisomycin. The proteins were fractionated on 8% SDS-PAGE, transferred to nitrocellulose, and immunoblotted using the monoclonal antibody 1D4-δ.

tabolising cells from arousing animals the turnover of coiled bodies may be greatly increased giving rise to a poorly structured morphology.

Coiled bodies are intimately associated with the nucleolus and, in fact, they were first described as "nucleolar accessory bodies" in neurons by Ramón y Cajal (1903). At the electron microscopic level, coiled bodies are frequently observed attached to the nucleolar periphery (Carmo-Fonseca *et al.*, 1993; Ferreira and Carmo-Fonseca, 1995) and, in some cases, they have even been detected inside the nucleolus (Malatesta *et al.*, 1994; Ochs *et al.*, 1994). Although coiled bodies are apparently deprived of rRNA, they contain some nucleolar proteins such as fibrillarin (Raska *et al.*, 1990, 1991), Nopp140, NAP57 (Meier and Blobel, 1994), and ribosomal protein S6 (Jiménez-Garcia *et al.*, 1994). Furthermore, it has been shown recently that overexpression of mutated forms of the human p80 coilin gene induces a disorganization of both the coiled body and the nucleolus, demonstrating that the two structures share either a common structural framework or a common assembly pathway (Bohmann *et al.*, 1995a). During adenoviral infection the maturation of ribosomal RNA is severely depressed, and the morphology of the nucleolus is drastically altered (Raskas *et al.*, 1970; Ledinko 1972; Puvion-Dutilleul and Christensen, 1993). However, there is no evidence for a direct interaction of the virus with the nucleolus, and because drugs that inhibit protein synthesis do affect the processing of rRNA in a similar way, it has been proposed that this viral effect simply reflects the suppression of host protein synthesis (Tooze, 1980). Taking into account the close association between the nucleolus and the coiled body, it is conceivable that the disassembly of coiled bodies may be related with the disorganization of the nucleolus induced by protein synthesis inhibition. Accordingly, coiled bodies are also disassembled in cells infected by herpes simplex virus 1, which makes only minimal use of the cellular RNA-splicing machinery but shuts down host RNA polymerase I activity and ribosomal RNA synthesis (Phelan *et al.*, 1993) (A. Phelan and M. Carmo-Fonseca, unpublished observations).

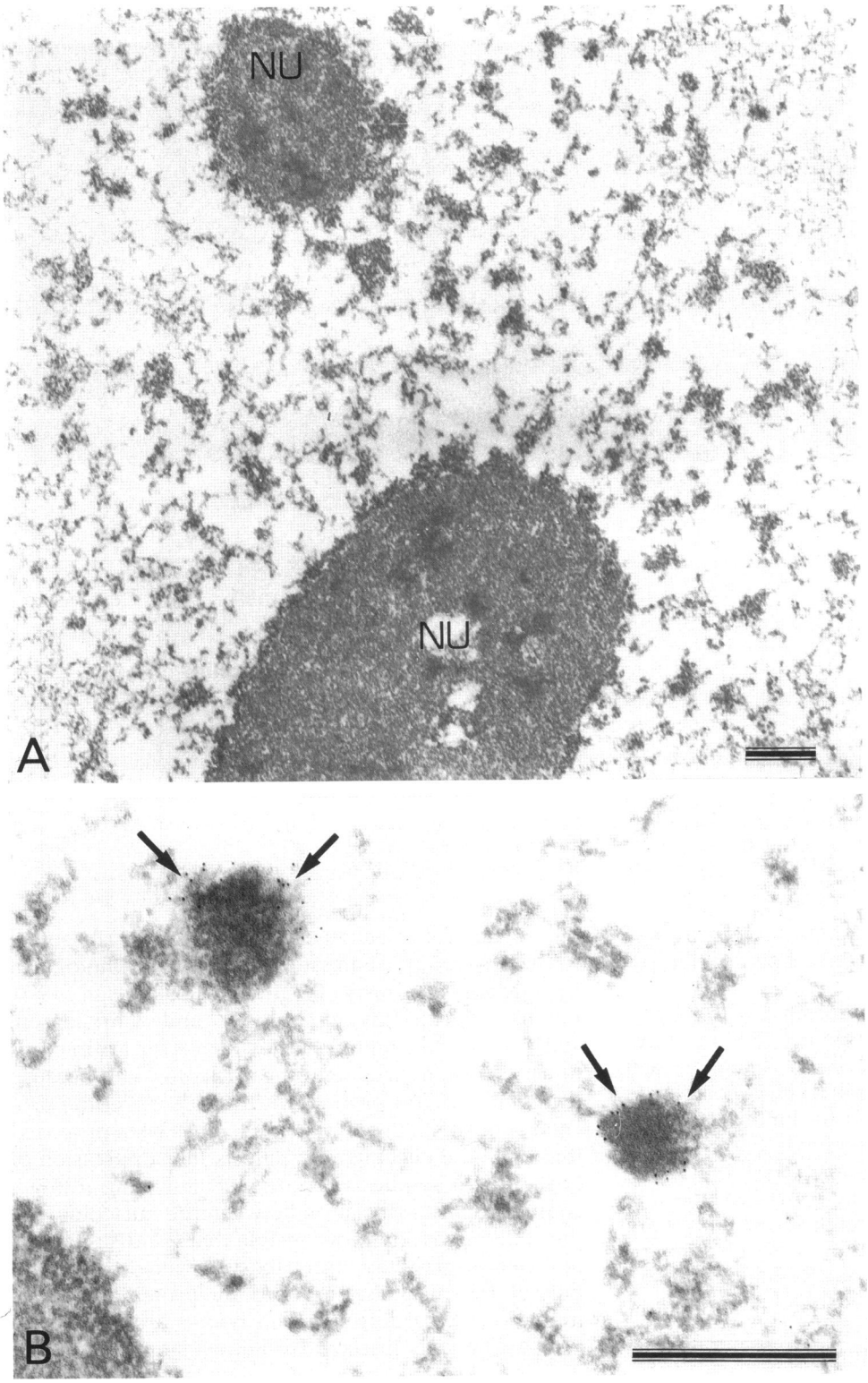

Figure 10. Immunoelectron microscopic localization of coilin in anisomycin-treated cells. HeLa cells were treated with anisomycin for 5 h and immunogold labelled using the anti-coilin monoclonal antibody 1D4-δ. Panel A shows that nucleoli (NU) have become compact, with few fibrillar centers. (B) The immunogold is specifically localized in dense, spheroidal structures ~0.3 μm in diameter (arrows). Bar, 0.5 μm.

The observed loss of coiled bodies in the nucleus of adenovirus-infected cells while viral RNA is being transcribed and exported to the cytoplasm at maximal rates (Bridge *et al.*, 1995) is in agreement with the idea that coiled bodies contain splicing snRNPs but do not represent major splicing sites. Although coiled bodies

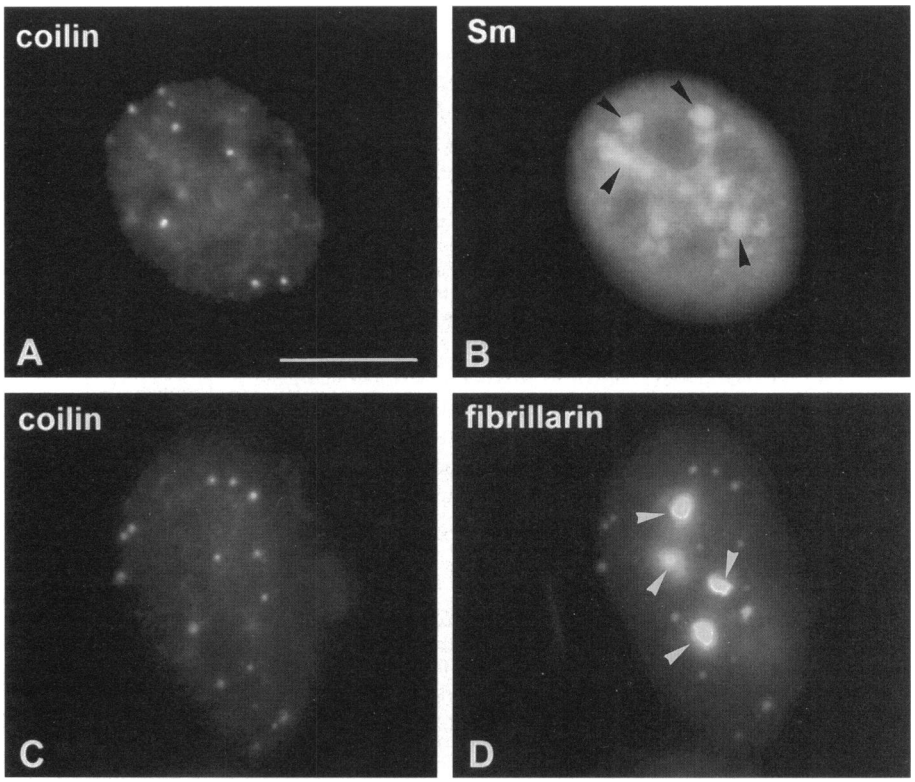

Figure 11. The coilin microfoci induced by anisomycin contain fibrillarin. HeLa cells were treated with anisomycin for 5 h and then double-labeled using either anti-coilin and anti-Sm antibodies (A and B) or anti-coilin and anti-fibrillarin antibodies (C and D). Arrowheads in B point to accumulation of Sm staining in enlarged speckles; arrowheads in D point to fibrillarin staining in nucleoli. Bar, 10 μm.

could be specialized sites where splicing of specific cellular pre-mRNAs takes place, the observation that they may also contain nonspliceosomal small RNAs such as the U3, U7, and U8 snRNAs (Wu *et al.*, 1993; Bauer *et al.*, 1994; Jiménez-Garcia *et al.*, 1994; Frey and Matera, 1995) suggests a more general function of the coiled body in the metabolism of snRNPs. Consistent with this view, it has been proposed recently that coiled bodies may play a role in post-transcriptional modification of snRNAs on base and sugar residues (Bohmann *et al.*, 1995b).

The finding that coiled bodies are very labile structures that require ongoing protein synthesis makes it unlikely that these are just sites where inactive snRNPs are passively stored. Rather, we favor the idea that coiled bodies play an active role in snRNA or snRNP metabolism, such as assembly, modification, or recycling. According to this concept, conditions that cause the disassembly of coiled bodies should interfere with snRNP structure and function. In fact, it is well known that the nucleus of Ad2-infected cells contains large amounts of partially spliced viral

Table 2. The effect of protein synthesis inhibitors on the number of coiled bodies per nucleus (N_{CB})

	Untreated	Anisomycin	Emetine	Cycloheximide
N_{CB}	% of cells	% of cells	% of cells	% of cells
0	18	71	56	54
1	26	20	26	28
2	37	8	15	15
3	15	1	3	3
4	3	0	0	0
5	1	0	0	0

Cells were treated with 100 μM anisomycin, 1 μM emetine, or 100 μM cycloheximide for 5 h. Coiled bodies were identified as brightly fluorescent foci with an apparent diameter of $\geq$0.8 μm. Microfoci ($\sim$0.3 μm) labeled by anti-coilin antibodies were not included in the analysis. The results are mean values from two independent experiments. A total of 3600 cells were analyzed ($\sim$450 per experimental group). Statistical analysis indicated that N_{CB} is altered by all drugs; treatment with emetine and cycloheximide produced similar frequency distributions, whereas treatment with anisomycin produced a significantly different distribution.

mRNAs that may correspond to accumulated aberrant intermediates (Sharp, 1984), and a possible cause for such inefficient processing could be incomplete snRNP recycling attributable to the absence of coiled bodies. On the other hand, treatment of cells with anisomycin induces a rapid and reversible disassembly of coiled bodies, with a parallel accumulation of splicing snRNPs in enlarged clusters of interchromatin granules. Because snRNPs have been shown previously to accumulate in interchromatin granules when transcription or splicing is inhibited (Carmo-Fonseca *et al.*, 1992; O'Keefe *et al.* 1994), one could speculate that in the absence of coiled bodies snRNPs are no longer correctly recycled to participate in the splicing reaction. Clearly, further experiments are required to address specifically whether snRNPs become functionally defective after disassembly of the coiled body.

ACKNOWLEDGMENTS

We acknowledge Professor David-Ferreira for support and Dr. João Ferreira for critical discussions. We are also grateful to Mr. João Romão for animal care facilities and to Dora Brito for help with electron microscopy. We thank the following researchers and their laboratories for generously providing materials used in this study: Dr. Eileen Bridge for adenoviruses, Dr. Eng Tan for anti-fibrillarin antibodies, Dr. Iain Mattaj for anti-Sm antibody Y12, and Dr. Walther van Venrooij for anti-Sm autoimmune serum and monoclonal antibody 7.13. This study was supported by grants from Junta Nacional de Investigação Científica e Tecnológica and EU (Human Capital and Mobility Program).

REFERENCES

Andrade, L.E.C., Chan, E.K.L., Raska, I., Peebles, C.L., Roos, G., and Tan, E.M. (1991). Human autoantibody to a novel protein of the nuclear coiled body: immunological characterization and cDNA cloning of p80-coilin. J. Exp. Med. *173*, 1407–1419.

Bauer, D.W., Murphy, C., Wu, Z., Wu, C.-H.H., and Gall, J.G. (1994). In vitro assembly of coiled bodies in *Xenopus* egg extracts. Mol. Biol. Cell *5*, 633–644.

Bauren, G., and Wieslander, L. (1994). Splicing of Balbiani ring 1 gene pre-mRNA occurs simultaneously with transcription. Cell *76*, 183–192.

Beyer, A.L., and Osheim, Y.N. (1988). Splice site selection, rate of splicing, and alternative splicing on nascent transcripts. Genes Dev. *2*, 754–765.

Billings, P.B., Allen, R.W., Jensen, F.C., and Hoch, S.O. (1982). Anti-RNP monoclonal antibodies derived from a mouse strain with lupus-like autoimmunity. J. Immunol. *128*, 1176–1180.

Bohmann, K., Ferreira, J., and Lamond, A.I. (1995a). Mutational analysis of p80 coilin indicates a functional interaction between coiled bodies and the nucleolus. J. Cell Biol. *131*, 817–831.

Bohmann, K., Ferreira, J., Santama, N., Weis, K., and Lamond, A.I. (1995b). Molecular analysis of the coiled body. J. Cell Sci. Suppl. *19*, 107–113.

Bouteille, M., Laval, M., and Dupuy-Coin, A.M. (1974). Localization of nuclear functions as revealed by ultrastructural autoradiography and cytochemistry. In: The Cell Nucleus, ed. H. Busch, New York: Academic Press, 3–71.

Bridge, E., Carmo-Fonseca, M., Lamond, A.I., and Pettersson, U. (1993). Nuclear organization of splicing snRNPs in adenovirus-infected cells. J. Virol. *67*, 5792–5802.

Bridge, E., Xia, D.X., Carmo-Fonseca, M., Cardinali, B., Lamond, A.I., and Pettersson, U. (1995). Dynamic organization of splicing factors in adenovirus-infected cells. J. Virol. *69*, 281–290.

Carmo-Fonseca, M., Ferreira, J., and Lamond, A.I. (1993). Assembly of snRNP-containing coiled bodies is regulated in interphase and mitosis: evidence that the coiled body is a kynetic nuclear structure. J. Cell Biol. *120*, 841–852.

Carmo-Fonseca, M., Pepperkok, R., Carvalho, M.T., and Lamond, A.I. (1992). Transcription-dependent colocalization of the U1, U2, U4/U6, and U5 snRNPs in coiled bodies. J. Cell Biol. *117*, 1–14.

Conover, W.J. (1980). Practical Nonparametric Statistics, 2nd ed., New York: John Wiley & Sons.

Fakan, S. (1994). Perichromatin fibrils are in situ forms of nascent transcripts. Trends Cell Biol. *4*, 86–90.

Ferreira, J., and Carmo-Fonseca, M. (1995). The biogenesis of the coiled body during early mouse development. Development *121*, 601–612.

Ferreira, J.A., Carmo-Fonseca, M., and Lamond, A.I. (1994). Differential interaction of splicing snRNPs with coiled bodies and interchromatin granules during mitosis and assembly of daughter cell nuclei. J. Cell Biol. *126*, 11–23.

Fey, E.G., Krochmalnic, G., and Penman, S. (1986). The nonchromatin substructures of the nucleus: the ribonucleoprotein (RNP)-containing and RNP-depleted matrices analyzed by sequential fractionation and resinless section electron microscopy. J. Cell Biol. *102*, 1654–1665.

Frey, M.R., and Matera, A.G. (1995). Coiled bodies contain U7 small nuclear RNA and associate with specific DNA sequences in interphase human cells. Proc. Natl. Acad. Sci. USA *92*, 5915–5919.

Gall, J.G., Tsvetkov, A., Wu, Z., and Murphy, C. (1995). Is the sphere organelle/coiled body a universal nuclear component? Dev. Genet. *16*, 25–35.

Grollman, AP. (1968). Inhibitors of protein biosynthesis. V. Effects of emetine on protein synthesis and nucleic acid biosynthesis in HeLa cells. J. Biol. Chem. *243*, 4089–4094.

Harlow, E., and Lane, D. (1988). Antibodies—A Laboratory Manual, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Horwitz, M.S. (1990). Adenoviridae and their replication. In: Virology, vol. 1, ed. B.N. Fields and D.M. Knipe, New York: Raven Press, 1679–1721.

Jiménez-Garcia, L.F., Segura-Valdez, M.L., Ochs, R.L., Rothblum, L.I., Hannan, R., and Spector, D.L. (1994). Nucleogenesis: U3 snRNA-containing prenucleolar bodies move to sites of active pre-rRNA transcription after mitosis. Mol. Biol. Cell *9*, 955–966.

Jiménez-Garcia, L.F., and Spector, D.L. (1993). In vivo evidence that transcription and splicing are coordinated by a recruiting mechanism. Cell *73*, 47–59.

Lafarga, M., Berciano, M.T., Andres, M.A., and Testillano, P.S. (1995). Effects of cycloheximide on the structural organization of the nucleolus and the coiled body in normal and stimulated supraoptic neurons of the rat. J. Neurocytol. (*in press*).

Lamond, A.I., and Carmo-Fonseca, M. (1993). The coiled body. Trends Cell Biol. *3*, 198–204.

Ledinko, N. (1972). Nucleolar precursor RNA and protein metabolism in human embryo kidney cultures infected with adenovirus 12. Virology *49*, 79–89.

LeMaire, M.F., and Thummel, C.S. (1990). Splicing precedes polyadenylation during *Drosophila* E74A transcription. Mol. Cell. Biol. *10*, 6059–6063.

Lerner, E.A., Lerner, M.R., Janeway, C.A., and Steitz, J.A. (1981). Proc. Natl. Acad. Sci. USA *78*, 2737–2741.

Malatesta, M., Zancanaro, C., Martin, T.E., Chan, E.K.L., Almaric, F., Luhrmann, R., Vogel, P., and Fakan, S. (1994). Cytochemical and immunocytochemical characterization of nuclear bodies during hibernation. Eur. J. Cell Biol. *65*, 82–93.

Meier, U.T., and Blobel, G. (1994). NAP57, a mammalian nucleolar protein with a putative homolog in yeast and bacteria. J. Cell Biol. *127*, 1505–1514.

Monneron, A., and Bernhard, W. (1969). Fine structural organization of the interphase nucleus in some mammalian cells. J. Ultrastruct. Res. *27*, 266–288.

Ochs, R.L., Stein, T.W., Jr., and Tan, E.M. (1994). Coiled bodies in the nucleolus of breast cancer cells. J. Cell Sci. *107*, 385–399.

O'Keefe, R.T., Mayeda, A., Sadowski, C.L., Krainer, A.R., and Spector, D.L. (1994). Disruption of pre-mRNA splicing in vivo results in reorganization of splicing factors. J. Cell Biol. *124*, 249–260.

Phelan, A., Carmo-Fonseca, M., McLauchlan, J., Lamond, A.I., and Clements, J.B. (1993). A herpes simplex virus type 1 immediate-early gene product, IE63, regulates small nuclear ribonucleoprotein distribution. Proc. Natl. Acad. Sci. USA *90*, 9056–9060.

Philipson, L. (1961). Adenovirus assay by the fluorescent cell counting procedure. Virology *15*, 263–268.

Pombo, A., Ferreira, J., Bridge, E., and Carmo-Fonseca, M. (1994). Adenovirus replication and transcription sites are spatially separated in the nucleus of infected cells. EMBO J. *13*, 5075–5085.

Puvion-Dutilleul, F., Bachellerie, J.P., Visa, N., and Puvion, E. (1994). Rearrangements of intranuclear structures involved in RNA processing in response to adenovirus infection. J. Cell Sci. *107*, 1457–1468.

Puvion-Dutilleul, F., and Christensen, M.E. (1993). Alterations of fibrillarin distribution and nucleolar ultrastructure induced by adenovirus infection. Eur. J. Cell Biol. *61*, 168–176.

Ramón y Cajal, S. (1903). Un sencillo método de coloracion selectiva del reticulo protoplasmico y sus efectos en los diversos organos nerviosos de vertebrados e invertebrados. Trab. Lab. Invest. Biol. *2*, 129–221.

Raska, I., Andrade, L.E.C., Ochs, R.L., Chan, E.K.L., Chang, C.-M., Roos, G., and Tan, E.M. (1991). Immunological and ultrastructural studies of the nuclear coiled body with autoimmune antibodies. Exp. Cell Res. *195*, 27–37.

Raska, I., Ochs, R.L., Andrade, L.E.C., Chan, E.K.L., Burlingame, R., Peebles, C., Gruol, D., and Tan, E.M. (1990). Association between the nucleolus and the coiled body. J. Struct. Biol. *104*, 120–127.

Raskas, H.J., Thomas, D.C., and Green, M. (1970). Biochemical studies on adenovirus multiplication. XVII. Ribosome synthesis in uninfected and infected KB cells. Virology *40*, 893–899.

Reimer, G., Pollard, K.M., Penning, C.A., Ochs, R.L., Lischwe, N.A., and Tan, E.M. (1987). Monoclonal antibody from (New Zealand Black × New Zealand White) F1 mouse and some human scleroderma sera target a Mr 34000 nucleolar protein of the U3-ribonucleoprotein particle. Arthritis Rheum. *30*, 793–800.

Roberts, R.J., Akusjarvi, G., Alestrom, P., Gelinas, R.E., Gingeras, T.R., Sciaky, D., and Pettersson, U. (1986). A consensus sequence for the adenovirus-2 genome. In: Adenovirus DNA, ed. W. Doerfler, Boston, MA: Martinus Nijhoff Publishing, 1–51.

Roth, M.B. (1995). Spheres, coiled bodies, and nuclear bodies. Curr. Opin. Cell Biol. *7*, 325–328.

Sharp, P.A. (1984). Adenovirus transcription. In: The Adenovirus, ed. H.S. Ginsberg, New York: Plenum Press, 173–204.

Siegel, S., and Castellan, N.J. (1988). Nonparametric Statistics for the Behavioral Sciences, 2nd ed., New York: McGraw-Hill.

Smetana, K., and Busch, H. (1974). The nucleolus and nucleolar DNA. In: The Cell Nucleus, ed. H. Busch, New York: Academic Press, 73–147.

Spector, D.L. (1993). Macromolecular domains within the cell nucleus. Annu. Rev. Cell Biol. *9*, 265–315.

Sproat, B.S., Lamond, A.I., Beijer, B., Neuner, P., and Ryder, U. (1989). Nucleic Acids Res. *17*, 3372–3386.

Tuma, R.S., Stolk, J.A., and Roth, M.B. (1993). Identification and characterization of a sphere organelle protein. J. Cell Biol. *122*, 767–773.

Tooze, J. (1980). DNA Tumor Viruses, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Wu, Z., Murphy, C., and Gall, J.G. (1994). Human p80-coilin is targeted to sphere organelles in the amphibian germinal vesicle. Mol. Biol. Cell *5*, 1119–1127.

Wu, Z., Murphy, C., Wu, C.-H.H., Tsvetkov, A., and Gall, J.G. (1993). Snurposomes and coiled bodies. Cold Spring Harbor Symp. Quant. Biol. *58*, 747–754.

Vazquez, D. (1979). Inhibitors of protein synthesis. Heidelberg, Germany: Springer-Verlag.

Zhang, G., Taneja, K.L., Singer, R.H., and Green, M.R. (1994). Localization of pre-mRNA splicing in mammalian nuclei. Nature *372*, 809–812.

Zhang, Y., and Schneider, R.J. (1993). Adenovirus inhibition of cellular protein synthesis and the specific translation of late viral mRNAs. Semin. Virol. *4*, 229–236.

Ziff, E.B. (1980). Transcription and RNA processing by DNA tumor viruses. Nature *287*, 491–499.