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The behavior of the coiled body in cells infected with adenovirus *in vitro*

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Abstract

The coiled body is a phylogenetically conserved nuclear organelle whose function is not known. Probes for detection of p80-coilin, an 80 kDa protein enriched in the coiled body, have made possible studies determining the behavior of the coiled body during the cell cycle, in proliferating cells, as well as reports suggesting some relationship of the coiled body to mRNA splicing and to the nucleolus. The objective of this study is to examine the distribution of p80-coilin and nucleolar proteins in cells infected with adenovirus *in vitro*. HeLa cells grown as monolayers were infected with successive dilutions of type 5 human adenovirus culture and fixed in methanol/acetone at different time points. Single and double indirect immunofluorescence was performed with human autoantibodies to p80-coilin, fibrillarin, NOR-90/hUBF, RNA polymerase I, PM-Scl, and To, as well as rabbit polyclonal serum to p80-coilin (R288) and mouse monoclonal antibody to adenovirus 72-kDa DNA-binding protein. Indirect immunofluorescence (IIF) with anti- p80-coilin antibodies showed that the usual bright dot-like coiled body staining pattern was replaced in infected cells by 1–5 clusters of tiny dots at the periphery of the nucleus. This phenomenon was first detected within 12 h of infection and affected more severely cells with increased length and load of infection. Cells subjected to heat shock presented no such alteration. Double IIF showed that cells with abnormal coiled body appearance expressed the viral 72-kDa DNA-binding protein. Nucleolar proteins RNA polymerase I and NOR-90/hUBF became associated with the p80-coilin-enriched clusters and were no longer detected in the nucleolus. Other nucleolar proteins, like PM-Scl and To, remained associated to the nucleolus and were not detected in the newly formed clusters. Fibrillarin had a heterogeneous behavior, being restricted to the nucleolus in some infected cells while in some others it was associated with the p80-coilin-enriched clusters. Thus our results showed that *in vitro* adenovirus infection induced radical redistribution of nucleolar and coiled body constituents into newly formed structures characterized by clusters of tiny dots in the periphery of the nucleus. The fact that three major proteins involved in rRNA synthesis and processing colocalized with p80-coilin in these clusters may bring additional support to the idea that the coiled body and p80-coilin may be implicated in functions related to the nucleolus.

Introduction

The coiled body (CB) is a conserved nuclear structure found in plants and a diverse array of animals. It was initially identified by a silver staining technique at the beginning of the century as the nucleolar accessory body by Ramon y Cajal [1]. Later it was described at the electron microscopic level as a round threaded non membrane-delimited structure measuring 0.2 to 1.0 μm in diameter [2–4].

Despite being ubiquitously present in nature, not much is known about its function. The identification of human autoantibodies with reactivity to p80-coilin, a protein highly enriched to the coiled body [5], has made possible new approaches to the study of this enigmatic nuclear element, i.e., indirect immunofluorescence and immunoelectron microscopy. Among the new information acquired by these techniques, we learned that the coiled body is a cell cycle-dependent structure, that it is formed in G1 after the assembly of nucleoli and usually dispersed in mitosis [6, 7]. It is more prominent and

numerous in cells under high proliferative status, both *in vitro* [6] and *in vivo* [8]. A number of macromolecules have been detected in the coiled bodies, such as U1, U2, U3, U4, U5, U6, and U7 snRNPs as well as their respective snRNAs [9–12]. This information has been interpreted as a suggestion that the coiled bodies might be involved in some sort of function related to mRNA splicing. However, not all splicing factors are detected in the coiled bodies and only a small fraction of the total snRNPs is located in the coiled bodies at any one time [13].

Another set of data seems to point to a link between the coiled body and the nucleolus. The original observations of Cajal already emphasized the close topographic relationship between these structures. Electron microscopic studies have confirmed these observations and identified fibrillar threads connecting both structures [14, 15]. Recently it has been shown that the CB may actually be inside the nucleolus in certain cancer cell lines [16] as well as in brown adipocytes and hepatocytes of hibernating dormice [17]. A series of nucleolar proteins are also found in the CB, such as fibrillarin, Nopp140, NAP57, and ribosomal protein S6 [9, 18–20]. A recent study has demonstrated that the expression of certain p80-coilin deletion mutants in HeLa cells causes a radical rearrangement of the nucleolar architecture and loss of RNA polymerase I activity [21].

Adenovirus infection has been shown to promote rearrangement of an array of nuclear macromolecules and organelles, including the spliceosomes [22, 23] and the nucleolus [24]. The present study aims at describing the behavior of the CB and its major protein, p80-coilin, in adenovirus infected cells. It also addresses the relationship between p80-coilin and nucleolar proteins in such infected cells.

Materials and methods

Cell culture and viral infection. HeLa S3 cells were grown on round coverslips in RPMI medium supplemented with 10% fetal calf serum, 2 mM glutamine at 37 °C in the presence of 5% CO_2. Adenovirus type 5 from a culture obtained from the Centers for Disease Control (Atlanta, GA) was activated by three successive passages in HeLa cells. Subconfluent cell cultures were infected with successive dilutions (1:1 to 1:20) of adenovirus type 5 in 100 μl serum-free inoculum. After 1 h adsorption at 37 °C, 10 ml culture medium was added and cells were incubated at 37 °C for different lengths of time up to 36 h. Coverslips were recovered every 3 h, washed in phosphate-buffered saline pH 7.2 (PBS) and fixed in methanol at –20 °C for 5 min and acetone at –20 °C for 2 min. Air-dried coverslips were kept at –20 °C until used in an immunofluorescence assay.

As a control for nonspecific cell injury, cells were subjected to heat shock by incubation at 42 °C for periods from 1 to 4 h before fixation in methanol/acetone as mentioned.

Indirect immunofluorescence (IIF). Human anti-p80-coilin serum Tk [5] and rabbit polyclonal anti-p80-coilin serum R288 [6] were used for labeling the coiled body. Viral infection was monitored with mouse monoclonal antibody to adenovirus 72-kDa DNA-binding protein [25]. Mouse monoclonal antibody RPN 1197 against 72-kDa heat shock protein from HeLa cells (Amersham, Buckinghamshire, England) was used to identify cells undergoing heat shock. Nucleolar antigens fibrillarin [26], To [27], PM-Scl [28], NOR-90/hUBF [29], and RNA polymerase I [30] were detected with human sera S4, LC , JO, and BB, respectively. These sera were previously characterized at the W. M. Keck Autoimmune Disease Center, the Scripps Research Institute, La Jolla, CA and kindly donated by Drs. E.M. Tan and E.K.L. Chan. Fixed coverslips were incubated with 20 μl primary antibody diluted 1:100 in PBS for 30 min at 37 °C in a moist chamber. For double IIF cells were incubated simultaneously with two primary antibodies. Secondary antibodies conjugated to fluorescein isothiocyanate (Sigma, Chemical Co, St Louis MO) or rhodamine (Caltag) were diluted 1:100 and 1:300 in PBS, respectively, and incubated with cells for 30 min at 37 °C in a moist chamber. Immunofluorescence was read in a Olympus BX50 microscope using a 40X objective and pictures were obtained with Kodak Tri-X pan 400 film using Olympus PM-30 automatic photomicrograph system.

Results

Effect of adenovirus infection on the distribution of p80-coilin. Abnormalities in the distribution of p80-coilin were first noted 12 h after infection and became progressively more conspicuous. Cultures infected for 24 h with 1:20 adenovirus dilution were felt to be the most appropriate to study since they showed the most striking changes without excessive cell degeneration. In infected cells, the usual IIF coilin staining pattern

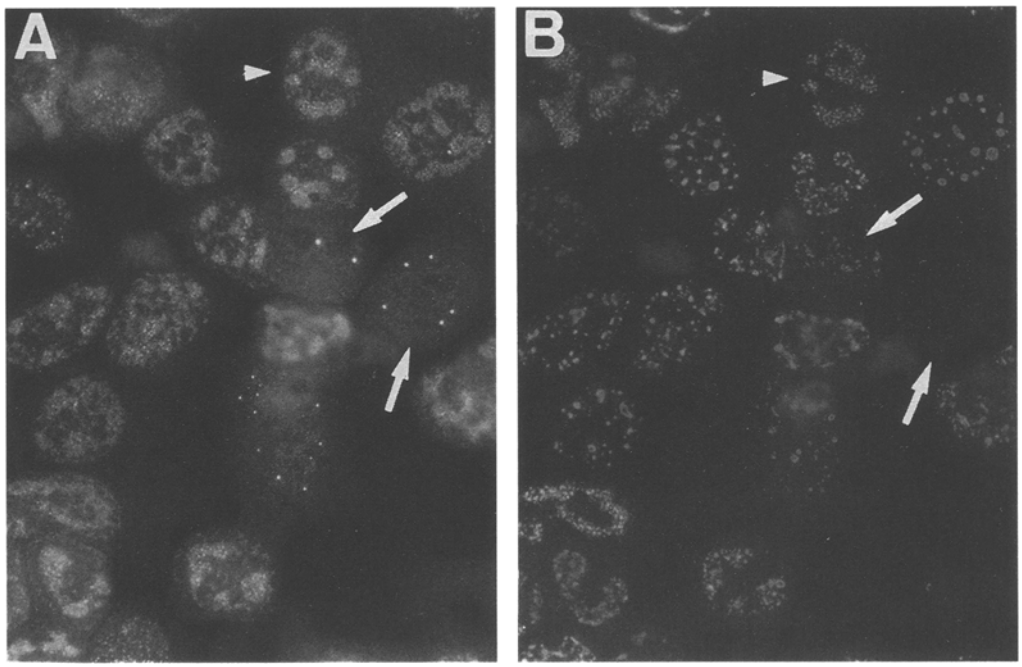

Figure 1. Double indirect immunofluorescence with HeLa cells infected with adenovirus. Panel A – cells stained with rabbit anti-p80-coilin serum R288. Panel B – cells stained with mouse monoclonal antibody RPN 1197 to adenovirus 72-kDa DNA-binding protein. Arrows depict cells with normal CB-like p80-coilin staining pattern and low or no viral protein expression. Arrowheads show colocalization of p80-coilin and adenovirus protein in clusters at the periphery of the nucleus.

represented by 1–6 bright scattered dots in the nucleoplasm was replaced by clusters of tiny dots in the nucleus. These clusters varied in number from 1 to 5 per cell and occurred around the central area of the nucleus (Figure 1A, arrowhead). In some cells, the staining was not represented by dots but rather by homogenous masses approximately 3–5 μm in diameter in the nucleus (Figure 1A). Double IIF demonstrated that cells with such abnormal p80-coilin staining expressed the viral 72-kDa DNA-binding protein (Figures 1A and B) while cells with usual CB-like pattern of p80-coilin distribution expressed no or only small amounts of the 72-kDa DNA-binding protein, possibly representing initial stages of infection (Figures 1A and B, arrows). Infected cells presented colocalization of p80-coilin and the adenovirus 72-kDa DNA-binding protein (Figures 1A and B, arrowhead). However, a closer look showed that the viral protein usually had a more discrete distribution inside the clusters as compared to p80-coilin.

To rule out the possibility that such a modification in p80-coilin distribution resulted from nonspecific injury, cells were studied after exposure to heat shock. After 2 h at 42° cells expressed the 72-kDa heat shock protein, as assessed by IIF but did not express the adenovirus 72-kDa DNA-binding protein. Heat shock-treated cells stained with anti-p80-coilin antibodies showed a gradual fading in CB staining (data not shown). In none of the time points after heat shock could the p80-coilin images obtained with adenovirus-infected cells be seen.

Colocalization of some nucleolar proteins with p80-coilin in adenovirus-infected cells. Double IIF showed that RNA polymerase I and NOR-90/hUBF shared a similar behavior, both of them redistributing to p80-coilin-stained clusters in infected cells (Figure 2). However, p80-coilin had a more discrete distribution inside the clusters, as compared to NOR-90/hUBF and RNA polymerase I, that frequently displayed a homogeneous staining within the clusters (Figure 2, arrows). In cells with a normal CB-like p80-coilin staining pattern (noninfected cells), RNA polymerase I and NOR-90/hUBF showed their usual nucleolar speckled staining (Figure 2, arrowheads). In an occasional cell, p80-coilin was distributed as scattered tiny dots (not grouped into clusters) and RNA polymerase I showed the regular nucleolar staining. This suggests that p80-

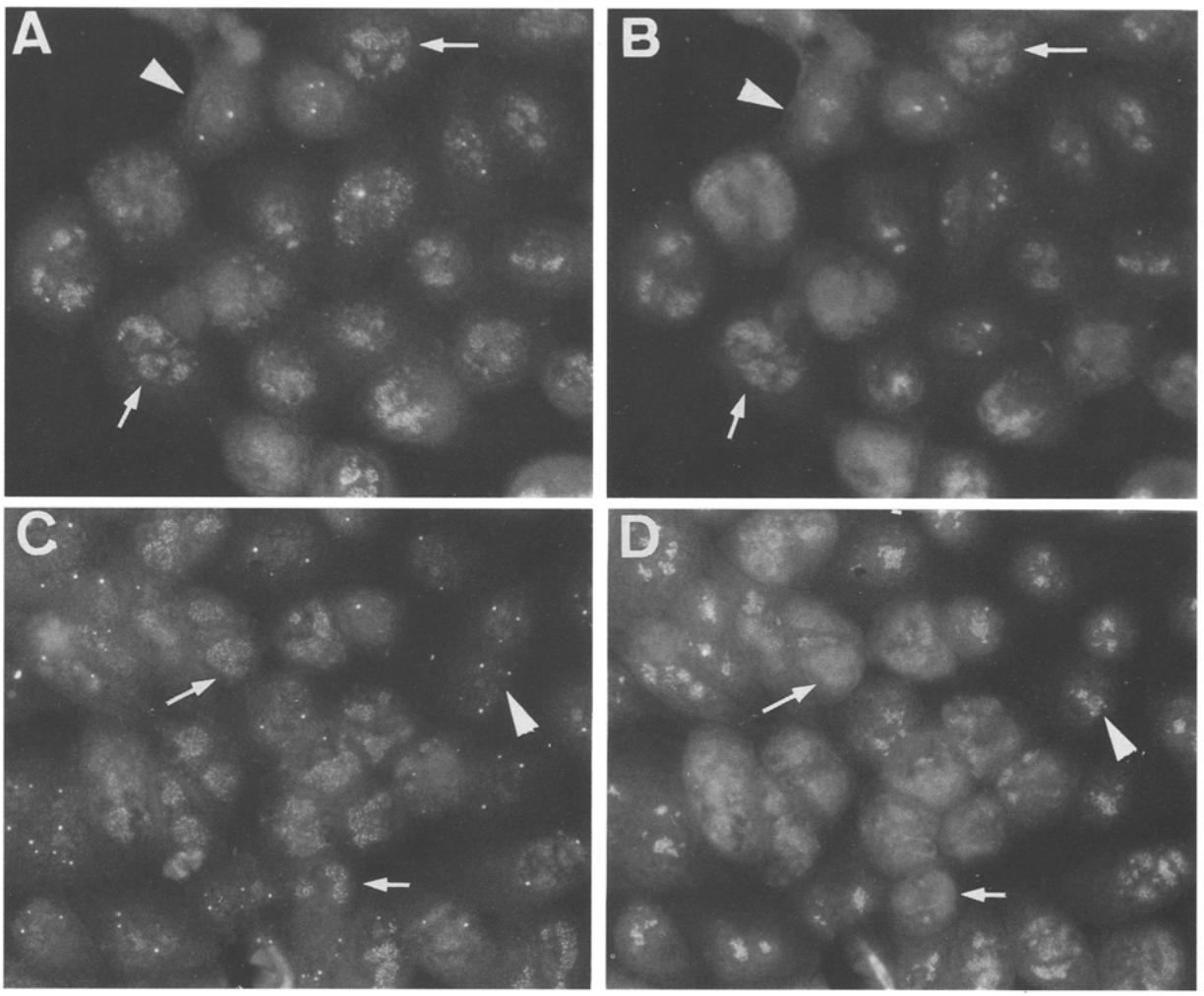

Figure 2. Double indirect immunofluorescence with HeLa cells infected with adenovirus. Panel A and C – cells stained with rabbit anti-p80-coilin serum R288. Panel B and D – cells stained with human autoantibodies to NOR-90/hUBF and RNA polymerase I, respectively. Arrows depict infected cells with colocalization of p80-coilin and NOR-90/hUBF (panels A and B) and RNA polymerase I (panels C and D).

coilin redistribution occurred prior to RNA polymerase I displacement from the nucleolus (data not shown).

A quite different behavior was observed for PM-Scl and To antigens, which did not migrate into p80-coilin-stained clusters in infected cells, but rather remained in the nucleolus or its remnants. These were frequently distorted or displaced to the periphery of the nucleus as if compressed by adjacent viral structures (Figures 3A and 3B, arrows). Fibrillarin presented a heterogeneous behavior in that some infected cells retained the usual nucleolar staining (Figures 3C and 3D, arrows) while some others depicted partial colocalization of fibrillarin and p80-coilin in extranucleolar sites (Figures 3C and 3D, arrowheads). In the latter situation, p80-coilin staining was represented by discrete speckles, and fibrillarin showed discrete speckles as well as areas of homogeneous staining.

Discussion

The present study showed a major redistribution in some of the macromolecules of the coiled body and the nucleolus in HeLa cells infected with adenovirus type 5. The usual staining pattern of p80-coilin, represented by 1–6 bright round spots in the nucleus, was gradually replaced by round clusters of many microdots. These clusters varied in number from 1 to

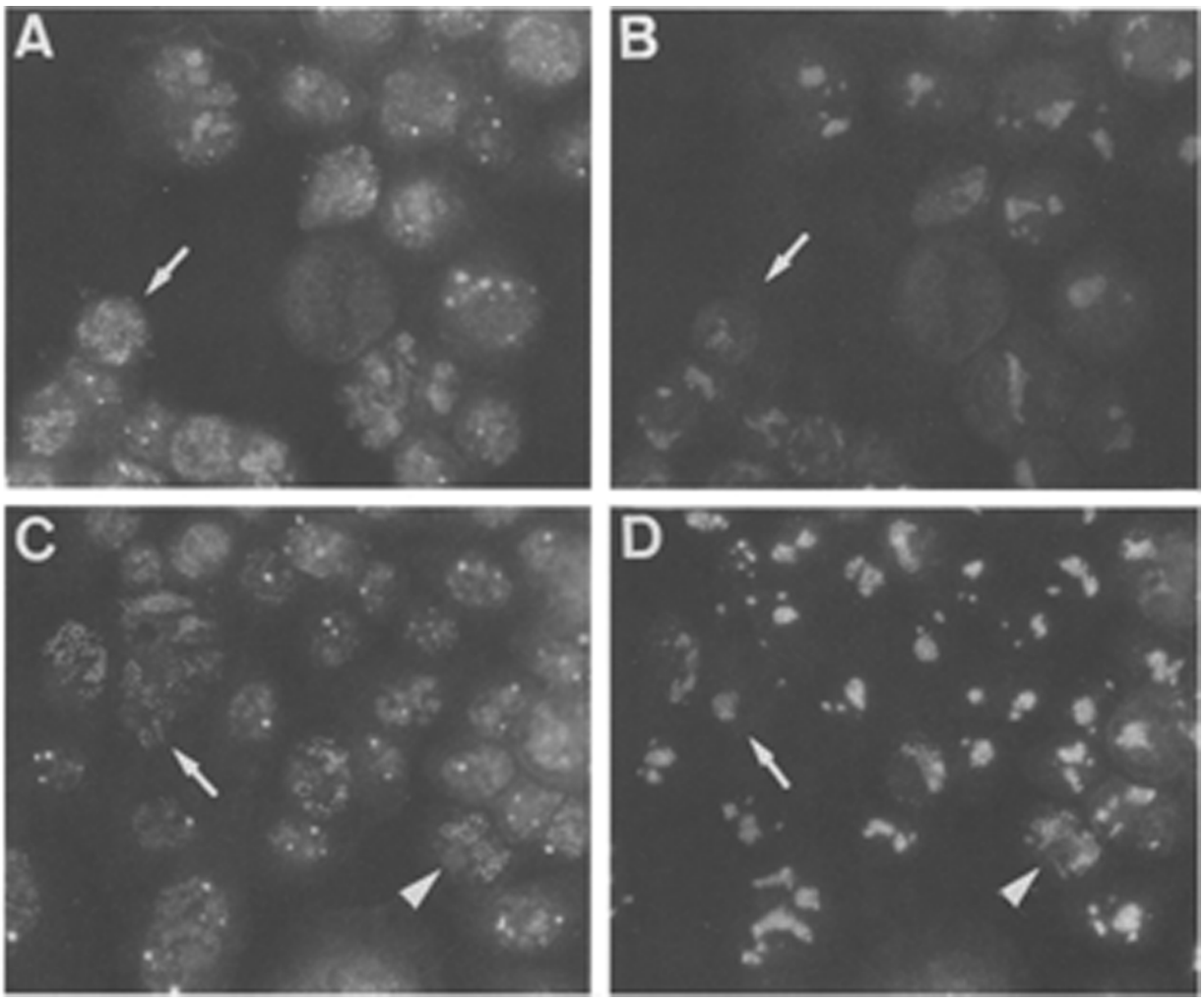

Figure 3. Double indirect immunofluorescence with HeLa cells infected with adenovirus. Panel A and C – cells stained with rabbit serum R288. Panel B and D, cells stained with human autoantibodies to PM-Scl and fibrillarin, respectively. Arrows depict infected cells with no colocalization of p80-coilin and PM-Scl (panels A and B) and fibrillarin (panels C and D). Arrowheads point to infected cells with partial colocalization of p80-coilin and fibrillarin.

5 per cell and were located around the central area of the nucleus. This phenomenon did not seem to result from nonspecific cell injury since heat shock-treated cells failed to show such behavior. Nucleolar proteins RNA polymerase I and NOR-90/hUBF (the upstream binding factor for RNA polymerase I) migrated from the nucleolus and colocalized into the newly formed clusters of p80-coilin-enriched microdots. Other nucleolar antigens, such as PM-Scl and To, remained in the nucleolus, while fibrillarin could be found both in the nucleolus and in some of the extranucleolar p80-coilin-enriched microdots.

The HeLa cell adenovirus infection model holds special interest because the virus uses the host enzymes to replicate its DNA and to transcribe and process its mRNA [31]. The distribution of adenovirus antigens in the cell has been well characterized by electron microscopy [22, 23]. Within 17 h of infection with adenovirus type 5, well-delineated compact fibrillar structures representing viral ssDNA accumulation sites are enclosed in a fibrillogranular network at the periphery of the nucleus (peripheral replicative zone) where the viral DNA is believed to be replicated and transcribed. In the center of the nucleus there is a large inclusion body representing the viral genome storage. Viral RNA

transcription occurs at clusters of interchromatin granules and at the peripheral replicative sites. The distribution of the host snRNPs evolves from the usual dispersed pattern (including coiled bodies and perichromatin granule-associated zones) in non-infected cells to a peripheral shell surrounding the central inclusion body in infected cells. Interestingly, these splicing components were not identified in the coiled bodies and in the perichromatin granule-associated zones in adenovirus-infected cells. Actually, no CBs and perichromatin granule-associated zones were observed in these cells [22].

Adenovirus infection in HeLa cells does not seem to severely disturb host DNA transcription but the mRNA is not transported to the cytoplasm and therefore protein synthesis is shut down [32]. It seems that host mRNA splicing is deeply inhibited and this is morphologically represented by the disappearance of perichromatin granule-associated zones in infected cells [22, 23]. Meanwhile, viral mRNA is successfully processed via host enzymatic machinery and transported to the cytoplasm. Adenovirus infection has also been shown to have profound inhibitor effects on the maturation of rRNA and assembly of ribosomes [24, 33].

The present data confirm recent findings by Rebelo et al. in which HeLa cells infected with adenovirus type 2 showed a gradual disappearance of the coiled body concomitant to the appearance of many p80-coilin-enriched microfoci throughout the nucleoplasm. These microfoci appeared in electron microscopy as 0.3 μm diameter homogeneous and dense structures containing p80-coilin and fibrillarin but not snRNPs or adenovirus RNA. In our study the p80-coilin-enriched microfoci were clearly grouped into 1-5 clusters of 3-5 μm in diameter at the periphery of the cell as opposed to the even distribution throughout the nucleoplasm reported by Rebelo et al. [34]. This difference may be related to peculiar variables in each study, such as the type of virus used and the time of infection in which observations were made. Rebelo et al. [34] suggested that the effects of adenovirus infection on the coiled body are related to blockage of host protein synthesis, since these effects could be reproduced by treatment of the cells with inhibitors of protein synthesis.

The p80-coilin-enriched microfoci triggered by adenovirus infection might correspond to the extranucleolar fibrillar spots described by Puvion-Dutilleul and Christensen [24]. These authors observed a drastic modification in the nucleolus of HeLa cells infected with adenovirus type 5, characterized by displacement of nucleolar chromatin from the interior of the nucleolus, compaction of the nucleolus with a predominance of the granular component, migration of the dense fibrillar component (DFC) to the periphery of the nucleolus, and disappearance of the fibrillar centers. Additionally, they observed extranucleolar fibrillar spots (0.15-0.3 μm in diameter) with similar morphological staining features with uranyl acetate as the DFC. These structures were often, but not always, enriched in fibrillarin as shown by immunoelectron microscopy.

Our findings confirm and extend the observations of Puvion-Dutilleul and Christensen [24] by showing that not only fibrillarin, but also other proteins usually associated with nucleolar fibrillar components, like RNA polymerase I (FC) and NOR-90/hUBF (FC and DFC), migrated from the nucleolus into extranucleolar sites. Furthermore, we were able to show that these extranucleolar sites were also enriched in p80-coilin and adenovirus 72-kDa DNA-binding protein. Although the significance of this phenomenon is not known, it is intriguing to observe that proteins involved in the synthesis and processing of rRNA (RNA polymerase I, NOR-90/hUBF, and fibrillarin) were sequestered from the nucleolus and colocalized with p80-coilin in the course of a process in which rRNA synthesis and mRNA processing and transport are severely disturbed. In that setting it is relevant that fibrillarin and silver-stained nucleolar proteins have been previously demonstrated in extranucleolar sites in various situations in which rRNA synthesis and maturation are disturbed, such as in intracellular injection of anti-RNA polymerase I antibodies [35], infection with herpesvirus [36], and treatment with low dose actinomycin D [14, 37].

The study of the coiled body under different physiological and pathological conditions may provide insights into the understanding of the nature and function of this intriguing structure. The demonstration of some splicing factors in the coiled body has been interpreted as a hint of its involvement in some aspect of this complex process. Some have suggested that the coiled body participates in the processing, storage or recycling of splicing factors [9, 38, 39]. Others have proposed that it may be responsible for the splicing of selected species of mRNA [10]. Another group of observations points to the involvement of the coiled body in functions related to the nucleolus. These include the morphological association of the coiled body and the nucleolus [2, 40], the frequent finding of intranucleolar coiled bodies in some cancer cell lines and in brown adipocytes of hibernating dormice [16, 17], the localization of nucleolar proteins

fibrillarin, Nopp140, NAP57, and ribosomal protein S6 in the coiled body [9, 14, 18–20], and the observation that overexpression of mutant forms of p80-coilin causes striking modifications in the nucleolus [20]. The present set of data brings further support in favor of some kind of relationship between the nucleolus and the coiled body in that it shows the colocalization of nucleolar proteins into newly formed structures enriched in p80-coilin. It is remarkable that the nucleolar proteins allocated to the p80- coilin-enriched microfoci were specifically those involved in rRNA transcription and processing, and may represent a link between the coiled body and p80-coilin to this major nucleolar function, independent of its putative participation in mRNA splicing.

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