Mutational Analysis of p80 Coilin Indicates a Functional Interaction between Coiled Bodies and the Nucleolus

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Abstract. Coiled bodies are conserved subnuclear domains found in both plant and animal cells. They contain a subset of splicing snRNPs and several nucleolar antigens, including Nopp140 and fibrillarin. In addition, autoimmune patient sera have identified a coiled body specific protein, called p80 coilin. In this study we show that p80 coilin is ubiquitously expressed in human tissues. The full-length human p80 coilin protein correctly localizes in coiled bodies when exogenously expressed in HeLa cells using a transient transfection assay. Mutational analysis identifies separate domains in the p80 coilin protein that differentially affect its subnuclear localization. The data show that p80 coilin has a nuclear localization signal, but this is not sufficient to target the protein to coiled bodies. The results indicate that localization in coiled bodies is not determined by a simple motif analogous to the NLS motifs involved in nuclear import. A specific carboxy-terminal deletion in p80 coilin results in the formation of pseudo-coiled bodies that are unable to recruit splicing snRNPs. This causes a loss of endogenous coiled bodies. A separate class of mutant coilin proteins are shown to localize in fibrillar structures that surround nucleoli. These mutants also lead to loss of endogenous coiled bodies, produce a dramatic disruption of nucleolar architecture and cause a specific segregation of nucleolar antigens. The structural change in nucleoli is accompanied by the loss of RNA polymerase I activity. These data indicate that p80 coilin plays an important role in subnuclear organization and suggest that there may be a functional interaction between coiled bodies and nucleoli.

The nucleus is a highly compartmentalized organelle. There is now evidence that protein and RNP factors involved in nuclear processes such as transcription, replication, pre-mRNA processing, and ribosome biogenesis are either partially or exclusively localized within discrete subnuclear domains or compartments (for review see Spector, 1993). In addition, several subnuclear compartments are known whose functions have not yet been identified. These include, inter alia, snRNP containing structures, such as coiled bodies and interchromatin granule clusters (for reviews see Gall, 1991; Brasch and Ochs, 1992; Lamond and Carmo-Fonseca, 1993; Spector, 1993; Bohmann et al., 1995) and PML bodies (Weis et al., 1994; Dyck et al., 1994; Koken et al., 1994). However, relatively little is known about how distinct nuclear compartments are formed without the use of membranes or about how specific antigens are selectively transported and recruited into these domains. It has been reported that the SR domain, a motif comprised of repeating serine-arginine dipeptides found in certain splicing factors, plays a role in subnuclear targeting to speckled structures, though not to coiled bodies (Li and Bingham, 1991). In the case of the nucleolus, the evidence points to protein targeting involving complex signals rather than simple peptide motifs (for review see Scheer and Weisenberger, 1994). Although it is still at an early stage, there is increasing interest in applying molecular and cell biological techniques to characterize subnuclear domains and hence dissect the functional organization of the nucleus.

The best studied example of a subnuclear compartment is the nucleolus which was identified more than one hundred and fifty years ago by several researchers including Valentin, Schleiden, and others (for a review see Franke, 1988). The nucleolus is now known to be the site of ribosomal RNA gene transcription, rRNA maturation, and ribosome subunit assembly (for a review see Gerbi et al., 1990). The nucleolus is a dynamic structure, whose assembly and integrity is dependent on transcription of the ribosomal DNA repeats. The nucleolus is itself a complex organelle and contains at least three different substructural elements, defined on morphological criteria as the fibrillar centers, dense fibrillar component, and granular component, respectively (for reviews see Jordan, 1991; Scheer and Weisenberger, 1994). A variety of nucleolar antigens are now known to partition differentially between these elements. For example, fibrillarin and nucleolin are proteins predominantly associated with the dense fibrillar component while RNA polymerase I is reported to concentrate in the fibrillar centers. Although the detailed picture remains somewhat controversial, it is widely believed...
that these separate structural elements of the nucleolus play distinct, though possibly overlapping, roles in the process of ribosome biogenesis.

In contrast with the nucleolus, very little is known about the function of coiled bodies. These are conserved subnuclear organelles found in both plant and animal cells which were first described in 1903 (Cajal, 1903). They were originally described as nucleolar accessory bodies since they are often located at the periphery of the nucleolus, particularly in neuronal cells. More recent studies have shown that coiled bodies share several antigens with the nucleolus, including fibribilgranin and Nopp140 (Raska et al., 1991; Meier and Blobel, 1994). However, they do not contain ribosomal RNA and therefore appear unlikely to play a direct role in ribosome biogenesis. Coiled bodies are one of the nuclear domains that contain splicing snRNPs and are components of the overall punctate pattern revealed by immunofluorescence studies using snRNP specific antibodies (Fakan et al., 1984; Pavion et al., 1984; Visa et al., 1993; for recent reviews see Spector, 1993; Bohmann et al., 1995). However, they do not contain certain non-snRNP splicing factors, such as SC-35 (Spector et al., 1991; Raska et al., 1991; Carmo-Fonseca et al., 1992) and do not accumulate nascent pre-mRNA (Fakan et al., 1984). Therefore, despite the presence of snRNPs, they are not believed to be major sites of pre-mRNA splicing. It is possible instead that they are involved in some aspect of snRNP maturation, transport or recycling. Since there is so little known about the coiled body it is also possible that it is involved in some other aspects of nuclear function. The metabolic role of the coiled body thus remains enigmatic.

An important advance in studying the coiled body came with the recent identification of a coiled body specific protein, termed p80 coilin (Andrade et al., 1991). This was discovered as a human autoantigen recognized by a rare class of patient autoimmune sera (Raska et al., 1991; Andrade et al., 1991). The p80 coilin cDNA has been cloned and sequenced (Andrade et al., 1991; Chan et al., 1994; Wu et al., 1994; Carmo-Fonseca et al., 1994). The deduced protein sequence shows no obvious homology to other proteins that might offer clues as to its function. Xenopus Sph I is the only protein known to date that is related to p80 coilin (Tuma et al., 1993). The Sph I protein localizes to sphere organelles in Xenopus oocytes. These are subnuclear domains specifically found in oocytes, which may be related to or even homologous with coiled bodies (Bauer et al., 1994; Wu et al., 1994; Gall et al., 1995).

In this study we present a mutational analysis of the human p80 coilin protein. Specifically, we use a transient transfection assay to characterize domains in the p80 coilin protein involved in its localization. The data point to an important role for p80 coilin in nuclear organization and suggest an unexpected functional interaction between coiled bodies and the nucleolus.

**Materials and Methods**

**Generation of anti-p80 Collin Antibodies**

A partial p80 coilin cDNA clone, isolated from a HeLa cDNA library (Stratagene, La Jolla, CA), in pBluescript II SK- (pKH8) was cloned into the His-tag E. coli expression vector pRSET B (Invitrogen, Leek, Netherlands) at the EcoRI restriction site. The His-tag coilin fusion protein was expressed in E. coli and purified using an Ni2+NTA agarose affinity column (Qiagen, Hilden, Germany) using conditions as recommended by the suppliers.

A peptide of the p80 coilin-coding region from amino acids 393-407 (RGWGREENFSWKGA) was chosen and synthesized on an 8-branched polystyrene core forming a Multiple Antigenic Peptide (MAP) (Tam et al., 1985).

Rabbits were injected with 400 μg of the His-tag coilin fusion protein or p80 coilin peptide in PBS emulsified with an equal volume of Freund's complete adjuvant (Sigma, St. Louis, MO). Booster injections were carried out 3.5, 7.5 and 10 wk later with 150 μg His-tag coilin fusion protein or p80 coilin peptide in Freund's incomplete adjuvant. Sera 204 (anti-fusion protein) and 116 (anti-peptide) were collected 4, 5, 7, and 9 wk later. Antisera from the fifth and the third bleed, respectively, were used in this study.

### Construction of Wild-Type and Mutant myc-tagged p80 Coilin Constructs in Eukaryotic Expression Vectors

A myc-tag (MEKLLISEDDL) (Evans et al., 1985) was cloned in frame with the full-length p80 coilin-coding region in Bluescript SK- (pBS 751.2A) using complementary DNA oligonucleotides flanked by BstEII and Ncol restriction sites to form the plasmid pKH16. This myc-coilin construct from pKH16 was cloned into an eukaryotic expression vector pSG5 (Green et al., 1988) to form the p80 coilin expression plasmid pKH17.

Mutant p80 coilin constructs were derived from plasmid pKH17 as follows: Unidirectional digestion with exonuclease III from the carboxy terminus was carried out as described in Henikoff (1984). Mutants N2, N4, and N5 containing amino acids 1-121, 215 and 234 of the p80 coilin sequence, respectively, were obtained with this method.

The mutants Δ5 and Δ7 were constructed using PCR techniques and contain amino acids 172-291 and 205-291, respectively.

The other mutants were constructed using restriction enzyme sites in the p80 coilin-coding region and standard recombinant DNA techniques. Mutant N1 contains amino acids 1-93, mutant N3 contains amino acids 1-291, mutant Δ4 contains amino acids 94-291, mutant C1 contains amino acids 94-576, mutant C2 contains amino acids 479-576. The structures of all mutant constructs were verified by DNA sequencing.

### Cell Culture and Transfection Assays

HeLa cells were grown on coverslips in Dulbecco's modified eagle's medium (Gibco BRL, Berlin, Germany) supplemented with 1% glutamine, 10% FCS and 100 U/ml penicillin and streptomycin (Gibco BRL). Transfections were performed after cells had reached ~50% confluency using the supplies for 6-24 h. Microinjection of plasmid DNA was performed using the A1S microinjection system as previously described (Anseroge and Pepperkok, 1988).

### Fixation and Immunofluorescence

Cells were washed in PBS and fixed for 10 min with 3.7% paraformaldehyde in CSK buffer (10 mM Pipes [pH 6.8], 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 2 mM EDTA) at room temperature. Permeabilization was performed with either 0.5% Triton X-100 or 0.2% SDS in CSK buffer for 15 min at room temperature.

Immunofluorescence staining and in situ hybridization with antisense oligonucleotide probes were performed as previously described (Carmo-Fonseca et al., 1992). All fluorescence microscopy was carried out using the EMBI, Compact Confocal Microscope (Stelzer et al., 1991). Excitation wavelengths of 476 nm (for fluorescein) and 529 nm (for Texas red) were used. The two channels were recorded independently, images were processed using Adobe Photoshop (Adobe Systems Inc., Mountain View, CA) and printed on a Color Ease PS Printer (Kodak, Eastman Kodak Company, Rochester, NY).

The following antibodies were used: rabbit anti-p80 coilin polyclonal serum 204/5 (dilution:1:300), rabbit anti-riptide antibody 116/3 (dilution:1:100), rabbit anti-Noppl40 RF12 (dilution:1:50) (Meier and Blobel, 1992), mAb 9E10 (dilution:1:500) (Evans et al., 1983), mAb anti-fibrillin 72B9 (dilution:1:2) (Reimer et al., 1987a), mAb anti-B+ 4G3 (dilution:1:2) (Habets et al., 1989), mAb anti-nucleolin 7G2 (dilution:1:1,000), mAb anti-
Visualization of Transcription Sites

Visualization of transcription sites was performed basically as previously described (Jackson et al., 1993; Wansink et al., 1993) on HeLa cells grown on coverslips and permeabilized with 0.05% Triton X-100 but without using agarose encapsulation. In control experiments α-amanitin (50 μg/ml) was present during the transcription reaction.

Visualization of RNA polymerase I transcription sites was performed essentially as described (Wansink et al., 1993) on HeLa cells 48 h after transcription with the plasmid encoding the Δ4 mutant construct. A subset of cells were microinjected with a combination of α-amanitin (50 μg/ml) and Bromo-UTP (100 mM) corresponding to ~5% of the cell volume and incubated at 37°C for 1 h before fixation. Microinjection was performed using the AIS microinjection system as previously described (Ansdor and Pepperkok, 1998).

In Vitro Expression and Detection of p80 Collin Protein

For protein analysis either HeLa whole cell lysates or nuclear extracts were used. Whole cell lysates were prepared from HeLa cells 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 0.1% bromophenol blue). HeLa nuclear extracts were prepared as described by Dignam et al. (1983).

In vitro transcription/translation was performed on construct pBS751.2A (p80 collin cDNA in Bluescript SK-) using TNT Capped Tetrahydrocyctylate Lysate System (Promega, Madison, WI). Proteins were separated by SDS-PAGE on 8% or 10% acrylamide gels and transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). The membranes were blocked and washed in 2% non-fat 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 horseradish peroxidase and detected using ECL Western blotting detection reagents (Amersham International plc, Buckinghamshire, England).

Electron Microscopy

Immuno-electron microscopy of transfected and control HeLa cells was performed using the pre-embedding technique. The cells were grown on coverslips and briefly extracted with 0.1% Triton X-100 in CSK buffer for 5 min in SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol and 0.1% bromophenol blue). HeLa nuclear extracts were prepared as described by Dignam et al. (1983).

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Results

p80 Collin Is a Ubiquitously Expressed Coiled Body Protein

Isolation of human cDNA clones encoding p80 collin has been reported previously (Andrade et al., 1991; Chan et al., 1994; Wu et al., 1994; Carmo-Fonseca et al., 1994). To determine the expression pattern of collin mRNA, a human multi tissue Northern blot was analyzed using 32P-labeled collin cDNA as a probe (Fig. 1 a). A single poly(A) mRNA species of ~2.8 kb was detected in all eight tissues examined. The size of this transcript is consistent with the size of the cDNA clones isolated. The ubiquitous expression pattern of collin mRNA corroborates our previous observations that p80 collin protein can be detected by Western blotting in all mammalian cell lines examined (Carmo-Fonseca et al., 1993 and other unpublished data). From these data we infer that collin is a constitutively expressed nuclear protein.

It has been shown previously that antibodies raised against recombinant collin protein expressed in E. coli specifically stain coiled bodies when used for immunofluorescence and detect the same 80-kD protein band on Western blots that is recognized by autoantiser that selectively labeled coiled bodies (Andrade et al., 1991; Carmo-Fonseca et al., 1994). This strongly suggests that the identified cDNA encodes the proposed coiled body-specific protein, p80 collin. Before proceeding with a mutational analysis of this protein, we performed additional experiments to identify rigorously that this cDNA indeed encodes p80 collin and not an immunologically related protein. First, an in vitro transcript of collin cDNA was prepared and translated in a rabbit reticulocyte lysate (Fig. 1 b). This yielded a major translation product that closely comigrated on SDS-PAGE with endogenous p80 collin protein from a HeLa cell nuclear extract (Fig. 1 b, lanes 1 and 2). The slightly faster migration of the in vitro product is likely due to the absence of posttranslational modifications that are not accurately reproduced in the reticulocyte lysate (Carmo-Fonseca et al., 1993).

Second, the cloned cDNA was transfected into HeLa cells and its localization determined using a transient expression assay (Fig. 2). The p80 collin cDNA was fused to an myc-epitope tag at its amino terminus to facilitate detection independently from endogenous p80 collin and expressed under the control of an SV40 early promoter (Fig. 2 a). When transiently transfected into HeLa cells, the myc-tagged protein was expressed, and, due to the extra amino acids of the epitope tag, was detected as a slightly slower migrating band than endogenous p80 collin when a Western blot from transfected cells was probed with an anti-collin antibody (Fig. 2 b). A monoclonal antibody specific for the myc-epitope labeled only the upper collin band, as expected, as well as the endogenous c-myc protein (Fig. 2 b). Immunofluorescence analysis using the same anti-myc monoclonal antibody showed that typically 20–40% of cells were transfected. Consistent with previous observations, untransfected cells are not labeled by the anti-myc antibody although it recognizes denatured c-myc on a Western blot (Fig. 2 c and other data not shown) (Evan et al., 1985). In all transfected cells myc staining was
nuclear, including several bright foci and a variable level of widespread nucleoplasmic labeling, excluding nucleoli (Fig. 2 c). Double-labeling experiments using anti-myc together with either anti-coilin or anti-fibrillarin antibodies confirmed that the bright foci colocalized with coiled bodies (Fig. 2 c). We conclude that the human p80 coilin cDNA encodes a ubiquitously expressed protein that localizes to coiled bodies.

**Mutational Analysis of p80 Coilin**

From the transfection studies using the full-length myc-tagged protein, it is apparent that the p80 coilin protein sequence includes sufficient information to direct localization in coiled bodies. Furthermore, we observe that even in cells expressing high levels of the transfected coilin protein, there is no apparent increase in the average number of coiled bodies per nucleus (Fig. 2 c and data not shown). We conclude that under these growth conditions the level of coilin protein is not the limiting factor for coiled body formation.

We have set out to use the transient transfection assay to map sequence elements in the p80 coilin protein that are required for localization in coiled bodies. Therefore, a series of amino- and carboxy-terminal deletion mutants of p80 coilin were constructed, each fused at its amino terminus to the myc-epitope (Fig. 3). Each mutant was transiently transfected into HeLa cells, expressed under the control of the same SV40 early promoter used for the full-length coilin construct, and its localization pattern determined by immunofluorescence using the anti-myc antibody (Fig. 4 and other data not shown).

As shown above, control transfection with the full-length construct shows nuclear staining and localization to coiled bodies (Fig. 4 wt). The coilin sequence includes two motifs at amino acid positions 107–112 and 181–198 that closely match the consensus sequence of the simple and bipartite nuclear localization sequence (NLS)\(^1\), respectively (for a review see Dingwall and Laskey, 1991). Comparison of mutants N1 and N2, and A7 and A5, shows that the presence of short peptides encoding the simple or the bipartite NLS motif, respectively, changes from a cytoplasmic to a nucleoplasmic localization (Fig. 4 and other data not shown). These data are consistent with both NLS motifs having a function in nuclear import. However, they may contribute differentially to the transport reaction. The N2 mutant that retains the simple NLS motif but lacks the bipartite NLS motif still shows exclusive nuclear localization (Fig. 4 N2). In contrast, the Δ5 mutant retaining the bipartite NLS motif but lacking the simple NLS motif shows only partial nuclear localization (Fig. 4 Δ5). A more detailed analysis of the role of the separate NLS motifs will be presented elsewhere.

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1. Abbreviations used in this paper: NLS, nuclear localization sequence; snRNP, small nucleus ribonucleoprotein particle.
Two mutants, N2 and C1, which showed an exclusive nuclear accumulation gave a homogeneous nucleoplasmic staining pattern, excluding nucleoli (Fig. 4, N2 and C1). Neither mutant showed any apparent localization in either coiled bodies or any other subnuclear domains. However, in the presence of these mutants coiled bodies containing endogenous p80 coilin and splicing snRNPs could still be detected by immunofluorescence (data not shown). We infer that both mutants have lost sequence information required for their assembly into coiled bodies. Interestingly, the N2 mutant has a carboxy-terminal deletion that removes all the protein sequence downstream from amino acid 122, whereas the C1 mutant lacks the amino-terminal ninety three amino acids. The two mutants therefore have an overlapping sequence of only twenty eight amino acids. Furthermore, between them the two mutants encompass the complete p80 coilin-coding sequence (Fig. 3). We conclude from this that the ability to localize into coiled bodies has a more complex sequence requirement than a simple signal akin to the NLS motif that is sufficient to target proteins into the nucleus.

In contrast with the N2 mutant described above, a smaller carboxy-terminal deletion that removes all the protein sequence downstream of amino acid 291, produces a strikingly different phenotype (Fig. 4 N3). In this case, the mutant protein accumulates in specific subnuclear domains. However, as will be documented below, these structures are not authentic coiled bodies (cf. Figs. 5 and 6). The dif-

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**Figure 2.** Transient expression of myc-tagged p80 coilin. (a) The full-length p80 coilin cDNA was fused in frame at its amino terminus with a peptide sequence derived from c-myc in the expression vector pSG5. The structure of the resulting expression construct (pKH17) is diagrammed. (b) Immunodetection of p80 coilin in whole cell lysates of untransfected (lanes 1 and 3) and transfected HeLa cells (lanes 2 and 4). Both the endogenous and myc-tagged (arrow) p80 coilin proteins were detected using the rabbit polyclonal anti-p80 coilin antiserum 204/5 (lanes 1 and 2). The monoclonal anti-myc antibody recognizes only the slower migrating p80 coilin band (lane 4) as well as the endogenous c-myc protein (lanes 3 and 4). (c) Immunofluorescence detection of transiently expressed myc-tagged p80 coilin in transfected HeLa cells. After transfection with pKH17, HeLa cells were double labeled with anti-myc and either anti-p80 coilin (upper panel) or anti-fibrillarin (lower panel) antibodies. Note that the same anti-myc monoclonal antibody that recognizes denatured c-myc on a Western blot does not label native c-myc in immunofluorescence microscopy. The labeling in the fluorescein (green staining- anti-myc) and Texas red (red staining- anti-p80 coilin and anti-fibrillarin) channels was recorded separately and subsequently overlaid to provide the images shown. Colocalization of both antibody staining patterns produces a yellow color. Corresponding Nomarski images are shown in parallel. As previously shown (Raska et al., 1991), we observe in addition to the strong nucleolar labeling a weak staining of coiled bodies by the anti-fibrillarin antibody in untransfected cells. Bar represents 10 μm.
A difference in localization between mutants N2 and N3 correlates with the presence of an additional 170 amino acids in N3 which include the bipartite NLS motif.

Another distinct phenotype was observed when mutants N4 and N5 were analyzed, both of which lack most of the protein sequence downstream of the bipartite NLS (cf. Fig. 3). Here a striking accumulation of the mutant protein was observed around the nucleoli (Fig. 4 N5 and data not shown). Interestingly, a similar phenotype of perinucleolar accumulation was seen with mutant Δ4 (Fig. 4 Δ4). This is a double deletion mutant, lacking both amino- and carboxy-terminal sequences, but including the internal region

Figure 3. Structure of p80 coilin deletion mutants. Cartoon indicates the structures of the various p80 coilin deletion mutants analyzed in this study. The numbers refer to the amino acid residues of wild-type p80 coilin remaining in each mutant. All constructs were fused at their amino-termini to the same myc-epitope tag and have been subcloned into the same expression vector used for the wild-type construct. The approximate positions of the simple (NLSa) and bipartite (NLSb) consensus motifs are indicated.

Figure 4. Transient expression of p80 coilin mutants in HeLa cells. Localization patterns of wild-type and mutant p80 coilin proteins were determined by immunofluorescence analysis in the confocal microscope using anti-myc antibodies on HeLa cells fixed 24–36 h after transient transfection with plasmids encoding the respective mutant constructs. Bar represents 2 μm.
that contains both the simple and bipartite NLS motifs (cf. Fig. 3). A detailed analysis of the Δ4 phenotype is presented below (cf. Figs. 7–10).

In summary, comparison of the p80 coilin deletion mutant structures and phenotypes indicates that localization to coiled bodies can be uncoupled from nuclear import. While the full-length p80 coilin protein accumulates in coiled bodies following transient transfection, none of the amino- or carboxy-terminal deletions analyzed above show localization in coiled bodies. The data indicate that localization to coiled bodies is not mediated by a single short sequence motif.

Deletion Mutant N3 Does Not Accumulate in Coiled Bodies

Only one mutant protein, N3, localizes in specific subnuclear bodies. To investigate the relation of the bodies containing N3 with coiled bodies, we characterized both their ultrastructural morphology and antigen composition (Figs. 5 and 6).

Electron microscopy was performed on HeLa cells that had been transiently transfected with deletion mutant N3. Pre-embedding immunoelectron microscopy with the anti-myc antibody was carried out to detect sites of accumulation of the mutant protein (Fig. 5 a). The immunogold labeling specifically accumulates around a dense granular nucleoplasmic body. This differs in morphology to the characteristic appearance of coiled bodies. Parallel immunogold labeling of coiled bodies in untransfected HeLa cells with anti-p80 coilin antibodies shows the typical bundle of coiled fibrillogranular threads (Fig. 5 b). Both structures vary in size and we observed N3 containing bodies up to 0.6 μm in diameter. Based on morphological criteria, we conclude that the N3 containing bodies are not coiled bodies.

Deletion Mutant N3 Forms Pseudo Coiled Bodies

We also examined the antigen composition of N3 containing bodies using immunofluorescence in the confocal microscope (Fig. 6). HeLa cells were transiently transfected with the N3 deletion mutant and double labeled with anti-myc and anti-fibrillarin antibodies (Fig. 6 a). In untransfected cells the anti-fibrillarin antibody labels nucleoli and smaller loci, some of which correspond to coiled bodies (Raska et al., 1991). In transfected cells, a heterogeneous picture is observed. Some cells show colocalization of N3 with fibrillarin in several small foci (Fig. 6 a, arrowheads). Other transfected cells show several large foci that are stained by the anti-myc antibody but stained only weakly or not at all with the anti-fibrillarin antibody (Fig. 6 a, arrows). In these cells the anti-fibrillarin antibody still stains nucleoli in an apparently normal pattern. The proportion of transfected cells showing this latter phenotype increased with time after transfection. This suggests that the N3 containing bodies increase in size from smaller precursors. If the smaller foci that contain fibrillarin are indeed precursors of the larger structures that enlarge with time, then either fibrillarin is lost as they increase in size, or more likely, they grow without recruiting additional fibrillarin. Thus, the larger structures may still contain small amounts of fibrillarin which might be either inaccessible or too dilute to be evident in the confocal overlay. As shown below, the larger N3 containing foci frequently show a ring-like staining pattern with anti-myc antibodies. In
combination with the dense structure revealed by electron microscopy, this indicates that the internal antigens in these bodies may be inaccessible to antibody binding.

Cells transfected with deletion mutant N3 were also analyzed with anti-nucleolin and anti-RNA polymerase I antibodies (data not shown). In both cases similar staining patterns were obtained in transfected and untransfected cells and we did not observe colocalization of either of these nucleolar antigens with N3 in either small or large N3 containing foci. However, another nucleolar antigen, Nopp140, showed a striking accumulation in both small and large N3 containing foci (Fig. 6 c). In cells which have accumulated large N3 foci, the majority of Nopp140 labeling seems to be transferred from nucleoli to the foci. Despite this reorganization of Nopp140, there is little or no sign of morphological change in the nucleoli of transfected cells as judged either by phase contrast or electron microscopy (Fig. 6 d and other data not shown).

Figure 6. Mutant N3 forms pseudo-coiled bodies. HeLa cells were transiently transfected with the plasmid encoding p80 coilin mutant N3, fixed 24–36 h after transfection and analyzed by indirect immunofluorescence with the following combinations of antibodies: (a) anti-myc monoclonal antibody (green) and anti-fibrillarin autoantibodies (red), arrowheads show small loci stained by both antibodies, arrows indicate large N3 containing bodies, predominantly labeled by the anti-myc antibody. (b) Corresponding Nomarski image of a. (c) Anti-myc monoclonal antibody (green) and anti-Noppl40 rabbit antibody (red). (d) Corresponding Nomarski image of c. (e) Anti-p80 coilin rabbit antibody (green) and anti-U2 snRNP B' monoclonal antibody (red), arrows indicate snRNP containing coiled bodies in untransfected cells, arrowheads indicate N3 containing foci which lack snRNP in transfected cells. (f) Anti-myc monoclonal antibody (green) and anti-p80 coilin peptide rabbit antibody (red). Bar represents 10 μm.
We also tested whether any snRNPs or protein splicing factors colocalize in the N3 containing foci (Fig. 6 e and other data not shown). In untransfected HeLa cells anti-snRNP antibodies show a typical punctate labeling pattern with some of the snRNPs concentrated in coiled bodies (Fig. 6 e, coiled bodies appear yellow in overlay, indicated by arrows). However, in cells expressing the N3 mutant protein, the N3 foci do not contain splicing snRNPs (Fig. 6 e, foci appear green in overlay, indicated by arrowheads). The non-snRNP protein splicing factor SC-35 is not present in either coiled bodies or N3 containing foci (data not shown).

The effect on endogenous coiled bodies of expressing the N3 mutant protein was examined by double labeling transiently transfected cells with anti-p80 colin and anti-myc antibodies (Fig. 6 f). For this experiment an anti-peptide antibody raised against a peptide in the carboxy-terminal region of p80 colin was used to detect endogenous p80 colin since this does not react with the N3 deletion mutant. We observe no colocalization of endogenous p80 colin in N3 foci. Furthermore, in transfected cells the anti-p80 colin antibody no longer labels prominent coiled bodies. In some cells p80 colin shows accumulation in clusters located at the periphery of the nucleolus (Fig. 6 f).

In summary, the N3 deletion mutant is shown to accumulate in a new form of subnuclear body. This is a dense structure which sequesters the nucleolar antigen Nop1p140 and may also contain low levels of fibrillarin but not other nucleolar antigens tested. It does not label with antibodies specific for snRNPs or splicing factors or endogenous p80 colin. We will refer to these N3 containing foci as pseudo-coiled bodies.

**Deletion Mutant A4 Promotes Redistribution of Fibrillarin in the Nucleolus**

Several mutant derivatives of p80 colin, including N4, N5, and A4, showed a striking interaction with the periphery of the nucleolus (cf. Fig. 4). We have investigated this phenotype in more detail by transiently transfecting HeLa cells with the A4 mutant construct and examining the consequent effect on localization of nucleolar and nucleoplasmic antigens, as determined by immunofluorescence studies in the confocal microscope (Figs. 7–9).

Double labeling with anti-myc and anti-fibrillarin antibodies revealed a marked relocation of fibrillarin within the nucleoli of transfected cells (Fig. 7). Specifically, fibrillarin migrated to the periphery of the nucleolus, forming a concentric internal ring within the surrounding peripheral staining of the A4 mutant protein. Little or no colocalization was observed between fibrillarin and the A4 protein. This experiment was repeated at different time points from 24 h to 60 h after transfection with the A4 mutant (Fig. 7, a–f). At 24 h after transfection the migration of fibrillarin to the periphery of the nucleolus was already apparent, although the A4 mutant still showed a significant level of nucleoplasmic staining (Fig. 7 a). At later times the A4 mutant protein became concentrated around the periphery of the nucleolus while fibrillarin frequently formed large aggregates, usually connected to the fibrillarin rings at the nucleolar periphery (Fig. 7, c and e). At later time points fibrillarin was more often detected in aggregates or clusters, rather than in rings. The simplest interpretation of these data is that in the presence of the A4 mutant fibrillarin is induced to migrate to the periphery of the nucleolus where it subsequently forms aggregates and clusters. We also note that cellular morphology changes in transfected cells at late time points. Beyond 60 h of transfection there is a decrease in the number of transfected cells visible, suggesting that the expression of the A4 construct may prove lethal.

When the A4 mutant construct was microinjected into HeLa cells, rather than transfected, the redistribution of fibrillarin and accumulation of A4 protein around the nucleolus was observed within 2.5–4 h after injection (Fig. 8 a and other data not shown). This rapid effect indicates that this is a direct consequence of the expression of the A4 mutant protein, rather than an indirect effect of the transfection assay or growth conditions of the cells. The specificity of this effect on the nucleolus is also supported by pulse-labeling cells with BrUTP 24 h after transfection to reveal sites of nascent transcription (Fig. 8 b). In both transfected and untransfected cells there is a similar level of incorporation of BrUTP in a typical pattern for RNA polymerase II (Jackson et al., 1993; Wansink et al., 1993) of several hundred small foci scattered throughout the nucleoplasm. This demonstrates that cells in which the A4 mutant protein clusters around the nucleolus still show similar levels of RNA-polymerase II transcription to control cells, at least at earlier stages after transfection. We also investigated whether RNA-polymerase I transcription was affected by expression of the A4 protein (Fig. 8, c–f). For this assay HeLa cells transiently transfected with the A4 mutant construct were subsequently microinjected with α-amanitin to preferentially inhibit transcription by RNA-polymerases II together with BrUTP to monitor sites of transcription activity (see Materials and Methods).

Staining with anti-fibrillarin antibody shows the typical perinucleolar reorganization of fibrillarin in A4 transfected cells (Fig. 8 c, arrows). Analysis of the sites of Bromo-UTP incorporation clearly identifies cells that have been microinjected (Fig. 8 d). As evident in the confocal overlay, untransfected cells show intense BrUTP labeling of nucleoli (Fig. 8 e, arrowheads), whereas the nucleoli of transfected cells have not incorporated BrUTP (Fig. 8 e, arrows). We conclude that expression of the A4 protein inhibits RNA-polymerase I transcription but does not prevent nuclear transcription outside of nucleoli.

**Mutant A4 Causes a Major Disruption of Nucleolar Architecture**

The effect of expressing the A4 mutant protein on other nucleolar and nucleoplasmic antigens was also examined (Fig. 9). Double-labeling A4 transfected cells with anti-RNA polymerase I and anti-fibrillarin antibodies showed that expression of A4 protein results in a relocation of RNA polymerase I (Fig. 9 a). In transfected cells RNA polymerase I accumulated with fibrillarin in the large aggregates but apparently not in the ring structures at the periphery of the nucleolus stained by anti-fibrillarin antibodies (Fig. 9 a, arrow). We did not detect RNA polymerase I colocalizing with the A4 mutant protein (data not shown). This phenotype in transfected cells (Fig. 9 a, arrowheads) differs markedly from control cells where RNA-polymerase I is clearly localized within the nucleoli.
A double labeling with anti-nucleolin and anti-fibrillarin antibodies showed that these antigens colocalized with one another in rings at the periphery of the nucleolus, but did not colocalize with the Δ4 protein around the nucleolus (Fig. 9b and other data not shown). The large aggregates of fibrillarin, which were characteristic of late time points after transfection, did not label with anti-nucleolin antibodies. In cells showing such fibrillarin aggregates some of the ring structures labeled with the anti-nucleolin antibody were only weakly stained with the anti-fibrillarin antibody (Fig. 9b and other data not shown). This suggests that expression of Δ4 mutant protein initially causes both fibrillarin and nucleolin to colocalize at the nucleolar periphery. However, subsequently these antigens separate when fibrillarin accumulates in large aggregates.

We detect at least some ribosomal RNA remaining within the nucleolus in transfected cells (Fig. 9c). In some transfected cells it also appeared that the level of cytoplas-
Figure 8. Effect of mutant Δ4 on transcriptional activity. (a) Cells were microinjected with the Δ4 mutant construct and fixed 4 h after injection. Cells were labeled with anti-fibrillarin autoantiserum (red) and monoclonal antimyc antibody (green). (b) Cells were transfected with the Δ4 mutant construct and fixed at 48 h after transfection. For the analysis of transcription sites cells were incubated with BrUTP for 15 min (see Materials and Methods) immediately before fixation and stained with anti-p80 coilin rabbit antibodies (green) and anti-Bromo-deoxyuridine monoclonal antibodies (red). (c-f) Cells were transfected with the Δ4 mutant construct and fixed 48 h after transfection. A subset of cells were microinjected with a combination of α-amanitin and BrUTP 1 h before fixation. (c) Cells were stained with anti-fibrillarin autoantiserum (red), arrows indicate transfected cells; (d) cells were stained with anti-Bromo-deoxyuridine monoclonal antibodies (green), e shows a confocal overlay of the images shown in c and d, arrow indicates an example of a transfected and injected cell, arrowhead indicates an example of an untransfected and injected cell, asterisk indicates an example of an untransfected and uninjected cell. f shows the corresponding Nomarski image for this field of cells. Bar represents 10 μm.

mic ribosomal RNA staining was reduced, consistent with the inhibition of RNA polymerase I transcription described above (Fig. 9 c, arrowhead). It is also apparent that the expression of Δ4 mutant protein causes a major change in nucleolar structure that can be seen by phase contrast microscopy (Fig. 9 d). While none of the above nucleolar antigens colocalize with the Δ4 mutant protein, double-labeling transfected cells with anti-myc and anti-Nopp140 antibodies showed that Nopp140 accumulated together with Δ4 mutant protein around the nucleolus (Fig. 9 e). It is interesting that the Nopp140 protein selectively localizes with both the Δ4 and the N3 mutant proteins, although these two mutants cause such markedly different phenotypes (cf. Figs. 6 c and 9 e).

Double-labeling experiments with anti-snRNP or anti-SC-35 antibodies, together with anti-p80 coilin antibodies that recognize both endogenous and mutant coilin proteins, showed that neither splicing snRNPs nor protein splicing factor SC-35 colocalize in structures surrounding the nucleolus (Fig. 9 f and data not shown). While the punctate snRNP labeling pattern appears generally intact in the transfected cells, endogenous coiled bodies containing snRNPs are no longer detected (Fig. 9 f, note loss of yellow foci in transfected cells indicated by arrowheads). A similar conclusion that endogenous coiled bodies are no longer present when the Δ4 mutant is expressed emerged from double-labeling studies with anti-p80 coilin and antimyc antibodies (data not shown).
The morphology of the structure formed by the Δ4 mutant protein that surrounds the nucleolus was analyzed at the ultrastructural level. HeLa cells transfected with the Δ4 mutant construct were immunolabeled using the pre-embedding technique and analyzed in the electron microscope (Fig. 10). As revealed by immunogold labeling, the Δ4 mutant protein concentrates in fibrillar structures emanating from the outer periphery of the nucleolar remnants. Under these extraction conditions little internal structure remains within nucleoli of transfected cells as compared with untransfected controls (Fig. 10, a and b). This is further evidence of the disruptive effect of the Δ4 mutant protein on nucleolar architecture. The EM pictures closely parallel the structures revealed in the previous immunofluorescence micrographs. As well as the fibrillar structures surrounding the nucleolus, the typical dense aggregates at the nucleolar periphery are clearly visible (Fig. 10 a, arrow).

In summary, we observe that expression of the Δ4 mutant protein causes a specific disruption of coiled bodies and nucleoli. The latter effect involves a major reorganization of nucleolar antigens, a rearrangement of nucleolar architecture, and the loss of RNA polymerase I activity.

Discussion
In this study we show that the human p80 coilin gene encodes an ubiquitously expressed nuclear protein that is...
able to correctly localize in coiled bodies when transiently expressed in HeLa cells. Mutational analysis of the p80 coilin protein demonstrates that nuclear localization can be uncoupled from localization in coiled bodies. Thus, the nuclear localization signal is not sufficient to target p80 coilin to coiled bodies. It is likely that one or both of the NLS consensus motifs identified in the coilin sequence are involved in nuclear targeting. Consistent with this, p80 coilin has been shown recently to interact with hSRP10, a human NLS receptor which binds to both simple and bipartite NLS motifs (Weis et al., 1995).

While the mutational analysis shows that the NLS motifs are not sufficient to target p80 coilin to the coiled body, this does not exclude that they may play some role in coiled body localization. In this regard it is interesting that the p80 coilin mutants which form aberrant subnuclear domains, corresponding to pseudo-coiled bodies and perinucleolar structures, appear to sequester the nucleolar protein Nopp140. This protein was originally identified as an NLS-binding factor and has been shown to shuttle between the nucleus and cytoplasm (Meier and Blobel, 1990, 1992, 1994). Since Nopp140 was also shown to be present in endogenous coiled bodies, it is possible that it interacts with one or both NLS motifs in p80 coilin. Further experiments are required to test whether this interaction occurs and to determine what role, if any, it might play in transport or localization of coilin within the nucleus.

The localization of proteins to coiled bodies could in principle be mediated by a simple sequence motif analogous to the NLS motifs that target many proteins into the nucleus. However, our mutational studies on p80 coilin argue against this possibility. Removal of either amino- or carboxy-terminal sequences of p80 coilin prevents localization in coiled bodies, even though the truncated proteins efficiently accumulated in the nucleus. These data are more consistent with protein localization to coiled bodies either involving a more complex or extended sequence motif or being a multi-step process, possibly requiring de novo assembly. In this regard, the mechanism of protein localization in coiled bodies might show more similarity to nucleolar targeting, which is also believed to require complex signals, rather than simple peptide motifs (for a review see Scheer and Weiseberger, 1994). Complex sequence requirements may therefore prove to be a common feature of subnuclear localization mechanisms.

It is interesting to compare the present data with recent
studies on the localization of human p80 coilin expressed in Xenopus oocytes (Wu et al., 1994). The full-length myc-tagged p80 coilin protein was shown to localize in sphere organelles. However, in the oocyte system, a carboxy-terminal deletion mutant, which removed protein sequence downstream of amino acid 102 in the coilin sequence, still localized to sphere organelles. This contrasts with our observations in somatic human cells, where a similar carboxy-terminal deletion mutant of human p80 coilin was not able to localize in coiled bodies. This difference in behavior could reflect alternative mechanisms of subnuclear localization between Xenopus and human cells or even between somatic cells and oocytes. Another possibility is that the coiled body and sphere organelles are related, but not directly equivalent structures and hence employ differential localization mechanisms.

It is striking that at least two novel types of subnuclear structure were formed when specific p80 coilin mutants were transiently expressed in HeLa cells. Neither of these structures are normally detected in untransfected cells. Formation of both the pseudo-coiled bodies and the perinucleolar structures is dependent upon expression of the mutant coilin proteins. This points to p80 coilin playing an important role in organizing subnuclear structure. A likely possibility is that wild-type p80 coilin is involved in the normal pathway of coiled body formation. The novel structures could therefore represent defective assembly pathways resulting from expression of the truncated coilin mutants which lack necessary sequence information. They could also represent accumulation of authentic assembly intermediates which normally are transient structures but here cannot proceed further along the normal pathway of coiled body formation because requisite functional domains are missing from the mutant proteins. We note that endogenous coiled bodies containing splicing snRNPs are absent from cells expressing these specific mutant proteins. This loss of endogenous coiled bodies may result from the dynamic nature of the structure coupled with the dominant inhibitory effect of the mutant proteins on de novo assembly. The data show that not only are the mutant proteins incorporated into novel structures but their expression simultaneously prevents the endogenous wild-type p80 coilin from localizing in coiled bodies.

One of the most unexpected results to emerge from this study is the effect of certain p80 coilin mutants on disrupting the architecture of the nucleolus. The mutants Δ4, N4, and N5, which each concentrate in fibrillar structures around the periphery of the nucleolus, promote a dramatic redistribution of nucleolar antigens. In particular, they cause segregation of antigens from the fibrillar components of the nucleolus. In addition, nucleolar function is also disrupted by expression of the Δ4 mutant protein as evidenced by the inhibition of RNA polymerase I activity and probably results in cell death as judged by the sharp decrease in the number of transfected cells visible at late time points. These dominant and specific effects point to an intimate functional interaction between nucleoli and coiled bodies. It is known that coiled bodies, which are also fibrillar structures, contain several nucleolar antigens, including fibrillarin and Nopp140 (Raska et al., 1991; Meier andBlobel, 1994), and are frequently observed at the periphery of the nucleolus (for a review see Bohmann et al., 1995). There are also several reports that document the presence of coiled bodies within nucleoli of breast carcinoma cells (Ochs et al., 1994) and in specialized cell types in hibernating dormice (Malatesta et al., 1994). Endogenous p80 coilin was also shown to cluster at the periphery of the nucleolus when transcription is inhibited by treating cells with actinomycin D (Carmo-Fonseca et al., 1992). A similar clustering of wild-type p80 coilin at the nucleolar periphery was observed in this study when endogenous coiled bodies were lost after the expression of the N3 mutant protein (cf. Fig. 6f). These previous observations all suggest that there is a relationship between coiled bodies and nucleoli. However, the present study is the first evidence that a coiled body specific antigen may play an important role in the structure and/or function of the nucleolus. Although coiled bodies contain splicing snRNPs and not ribosomal RNA, these observations strongly suggest that there may be some common assembly, transport or other process that is important for both structures. Such a common process may involve antigens such as fibrillarin and Nopp140 which are present in both coiled bodies and nucleoli or the nucleolar snoRNP U3 which has also recently been detected in coiled bodies (Raska et al., 1991; Meier andBlobel, 1994; Jimenez-Garcia et al., 1994).

A major goal for future studies remains the identification of the functional role of the coiled body. Based on the present observations, a better understanding of the molecular interactions between coiled bodies and nucleoli may provide important clues in this direction. For example, the present data indicate that the expression of the p80 coilin mutant Δ4 can inhibit ribosomal RNA transcription. It will be interesting to determine whether this also affects ribosomal RNA maturation or ribosome assembly and in parallel whether assembly or function of splicing snRNPs is impaired. It is also possible that this could produce new insights into the physiological role of the nucleolus beyond its known function in ribosomal RNA synthesis and ribosome production.

The authors are grateful to Professor Dr. U. Scheer for human autoantibodies specific for fibrillarin and RNA-polymerase I; Professor G. Dreyfuss for monoclonal anti-nucleolin antibodies; Dr. U. T. Meier for rabbit anti-Noppl40 antibodies; Professor W. van Venrooij for monoclonal anti-U2 snRNP Bα antibodies; and Professor T. Maniatis for monoclonal anti-SC-35 antibodies and Dr. G. Evan for monoclonal anti-myc antibodies. We thank Rainer Saffrich for help with the microinjection experiments. We thank Cinzia Calvio and Niovi Santama for helpful comments during the preparation of the manuscript.

J. Ferreira was supported by short term fellowship of the Calouste Gulbenkian foundation.

Received for publication 16 June 1995 and in revised form 2 August 1995.

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