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.

T HE 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-argine 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

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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 fibrillarin 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; Puvion 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 SphI 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 Coilin 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$_6$-tag E. coli expression vector pRSET B (Invitrogen, Leek, Netherlands) at the EcoRI restriction site. The His$_6$-coilin fusion protein was expressed in E. coli and purified using an Ni-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 (RGWGREENLFSWKGA) was chosen and synthesized on an 8-branched polylysine core forming a Multiple Antigenic Peptide (MAP) (Tam et al., 1985).

Rabbits were injected with 400 μg of the His$_6$-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, and 10 wk later with 150 μg His$_6$-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, 8, 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 (MEQKLISEEDL) (Evan 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 NcoI 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/μl penicillin and streptomycin (Gibco BRL). Transfections were performed after cells had reached ∼50% confluency using Dotap (Boehringer Mannheim, Germany) and transfection conditions as recommended by the supplier for 6–12 h. Microinjection of plasmid DNA was performed using the AIS microinjection system as previously described (Ansorge 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 MgCl$_2$, 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 EMBL 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 (dilution1:300), rabbit anti-peptide antibody 116/3 (dilution1:100), rabbit anti-Nopp140 RF12 (dilution1:50) (Meier and Blobel, 1992), mAb 9E10 (dilution1:500) (Evan et al., 1985), mAb anti-fibrillarin 72B9 (dilution1:2) (Reimer et al., 1987a), mAb anti-B″ 4G3 (dilution1:2) (Habets et al., 1989), mAb anti-nucleolin 7G2 (dilution1:1,000), mAb anti-

SC35 (dilution1:50) (Fu and Maniatis, 1990), mAb anti-Bromo-deoxyuri-
dine (dilution1:20) (Boehringer, Mannheim, Germany), human antiserum
S4 anti-fibrillarin (dilution1:150) (Reimer et al., 1987a), and human anti-
serum S18 anti-RNA polymerase I (dilution1:100) (Reimer et al., 1987b).

In situ hybridization with biotinylated anti-28S rRNA 2'-O-alkyl-oli-
goribonucleotides (5'-AIAICCAAUCCUUAUdT-3'), synthesized as de-
scribed by Lamond and Sproat (1994), was performed as previously de-
scribed (Carmo-Fonseca et al., 1992).

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 us-
ing 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
transfection 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-dUTP (100 mM) corresponding to ~5% of the cell volume
and incubated at 37°C for 1 h before fixation. Microinjection was per-
formed using the AIS microinjection system as previously described (An-
sorge and Pepperkok, 1988).

Northern Blot Analysis

The human multiple tissue Northern blot (Clonetech, Palo Alto, CA) was
probed with the full-length cDNA probe of p80 coilin that was radiola-
beled by random priming (Feinberg and Vogelstein, 1983). Hybridizations
(50% formamide, 5× Denhardt's, 5× SSC, 20 mM NaPO₄ pH 6.5, 0.1%
SDS, 200 μg/ml herring sperm DNA at 42°C) and washes (0.1× SSC, 0.1%
SDS at 65°C) were performed under high stringency.

In Vitro Expression and Detection of p80 Coilin 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-mercap-
toethanol, 10% glycerol and 0.01% bromophenol blue). HeLa nuclear ex-
tracts were prepared as described by Dignam et al. (1983).

In vitro transcription/translation was performed on construct pBS751.2A
(p80 coilin cDNA in Bluescript SK-) using TNT Coupled Reticulocyte Ly-
sate 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
(Fey et al., 1986) containing 0.1 mM PMSF, for 30–60 s on ice, and fixed in
3.7% paraformaldehyde in CSK buffer for 30 min at room temperature.
After fixation the cells were further extracted with 0.5% Triton in CSK
buffer for 30 min, washed three times for 10 min with PBS. The cells were
incubated with either mAb anti-myc 9E10 diluted 1:500 or rabbit anti-coi-
lin antibody 204/5 diluted 1:250 in PBS, for 1 h at room temperature and
washed three times for 10 min in the same buffer. Cells stained with mAb
anti-myc 9E10 were incubated with an intermediate layer of rabbit-anti-
mouse antibodies (Dianova, Hamburg, Germany). Cells were subsequently
incubated with secondary antibodies coupled to either 15-nm or 5-nm gold
particles (Amersham International plc) for 1 h at room temperature. Af-
ter washing three times with PBS, cells were fixed in 2% glutaraldehyde in
PBS overnight, postfixed in 0.5% OsO₄, contrasted with 0.2% tannic acid
and 0.5% uranyl acetate/1% phosphotungstic acid, and embedded in
Epon as described by Langanger et al. (1984). Samples were examined
with a Philips EM 301 electron microscope operated at 80 kV.

Results

p80 Coilin Is a Ubiquitously Expressed Coiled Body Protein

Isolation of human cDNA clones encoding p80 coilin 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 coilin mRNA, a hu-
man multi tissue Northern blot was analyzed using ³²P-
labeled coilin 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 expres-
sion pattern of coilin mRNA corroborates our previous
observations that p80 coilin 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 coilin is a constitutively ex-
pressed nuclear protein.

It has been shown previously that antibodies raised
against recombinant coilin protein expressed in E. coli spe-
cifically stain coiled bodies when used for immunofluores-
cence and detect the same 80-kD protein band on Western
blots that is recognized by autoantisera that selectively la-
bel 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
coilin. Before proceeding with a mutational analysis of this
protein, we performed additional experiments to identify
rigorously that this cDNA indeed encodes p80 coilin and
not an immunologically related protein. First, an in vitro
transcript of coilin 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 coilin 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 ac-
curately 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 ex-
pression assay (Fig. 2). The p80 coilin cDNA was fused to
an myc-epitope tag at its amino terminus to facilitate de-
tection independently from endogenous p80 coilin and ex-
pressed 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 coilin when a
Western blot from transfected cells was probed with an
anti-coilin antibody (Fig. 2 b). A monoclonal antibody
specific for the myc-epitope labeled only the upper coilin
band, as expected, as well as the endogenous c-myc pro-
tein (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)
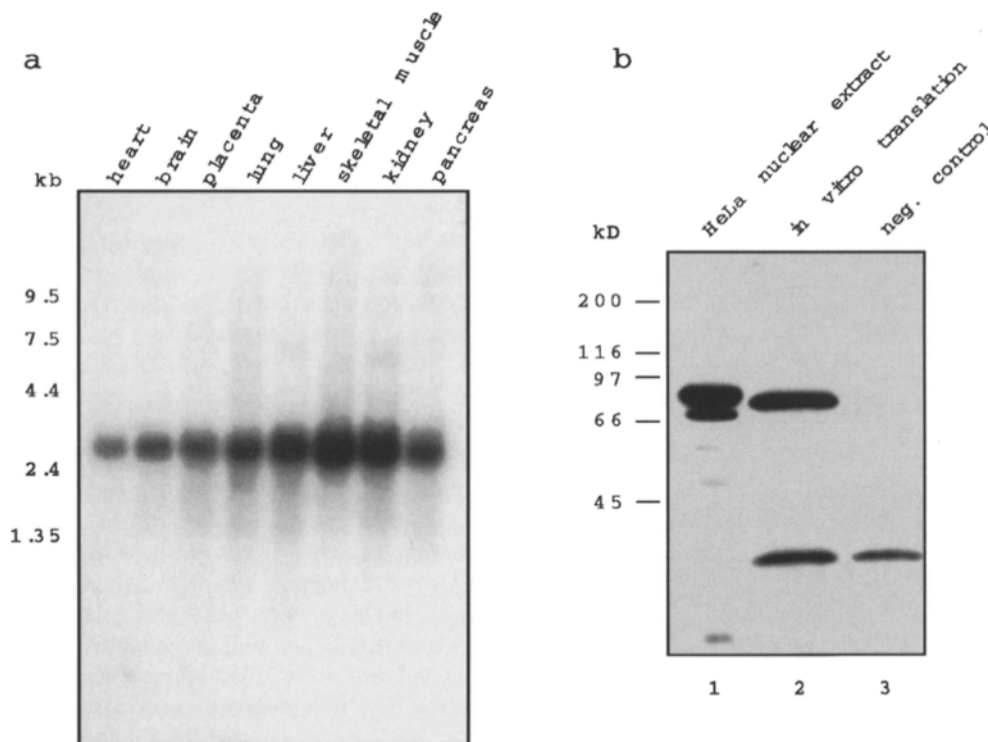(Evan et al., 1985). In all transfected cells myc staining was

Figure 1. Expression of p80 coilin. (*a*) A filter containing poly A+ RNA from eight different human tissues was probed with ^{32}P-labeled human p80 coilin cDNA. A single transcript of $\sim$2.8 kb was detected in all tissues. (*b*) Immunodetection of endogenous or in vitro translated p80 coilin. In vitro translation of human p80 coilin cDNA by rabbit reticulocyte lysate was performed in a coupled in vitro transcription/translation assay. (Lane *1*) HeLa cell nuclear extract. The lower products are most likely proteolytic cleavage products of p80 coilin and vary in intensity between different nuclear extract preparations. (lane *2*) Reticulocyte lysate after in vitro translation of p80 coilin mRNA. (lane *3*) Reticulocyte lysate control after in vitro translation in the absence of exogenous mRNA. The position of protein size markers run in parallel are indicated. The filter was probed with rabbit polyclonal anti p80 coilin antiserum 204/5.

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 Δ7 and Δ5, 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 $\Delta5$). 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.

a

rabbit β-globin
intron II

p80 coilin cDNA

SV40 early
promotor

myc-tag

SV40 polyA
site

b

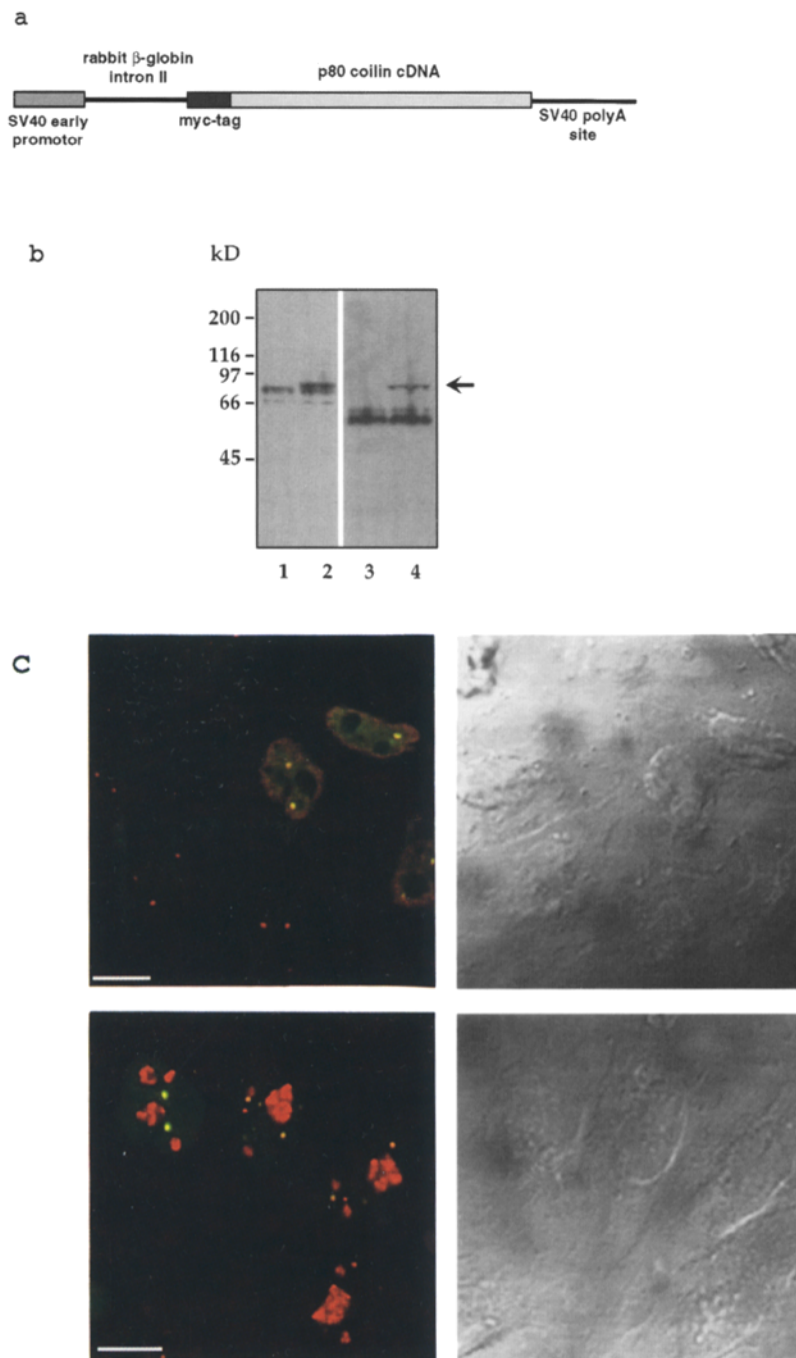kD

200 –
116 –
97 –
66 –

45 –

1   2   3   4

c

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.

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 istrikingly 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-
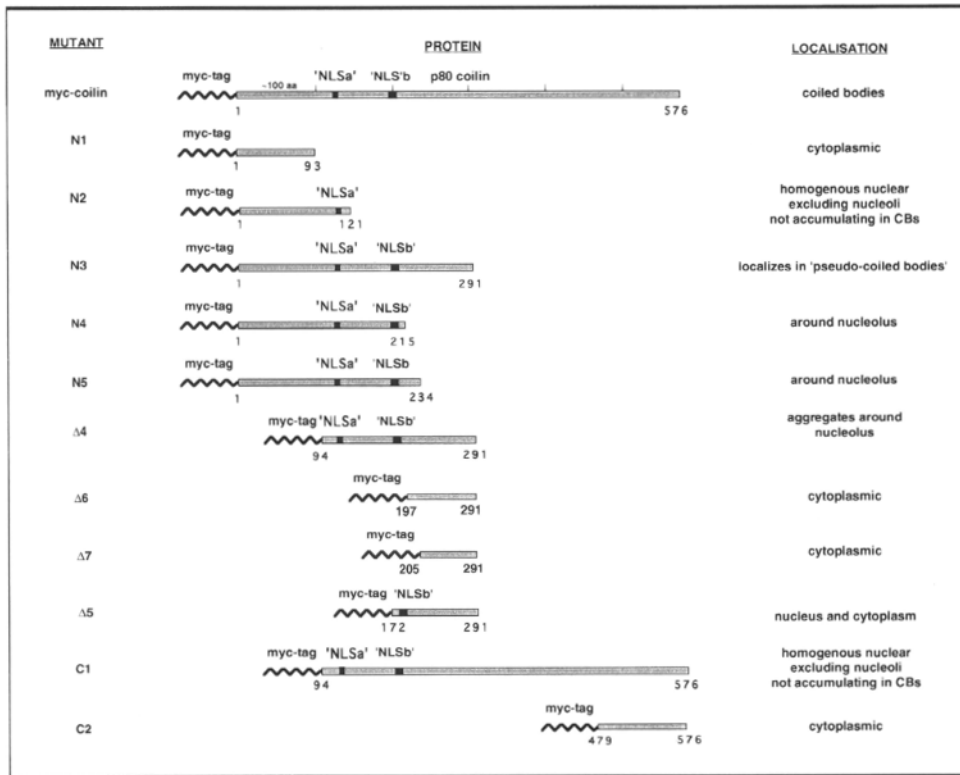
Figure 3. Structure of p80 coilin deletion mutants. Cartoon indicates the structures of the various p80 coilin deletion mutants analyzed in this study. The numbers refer to the amino acid residues of wild-type p80 coilin remaining in each mutant. All constructs were fused at their amino-termini to the 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.

ference 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
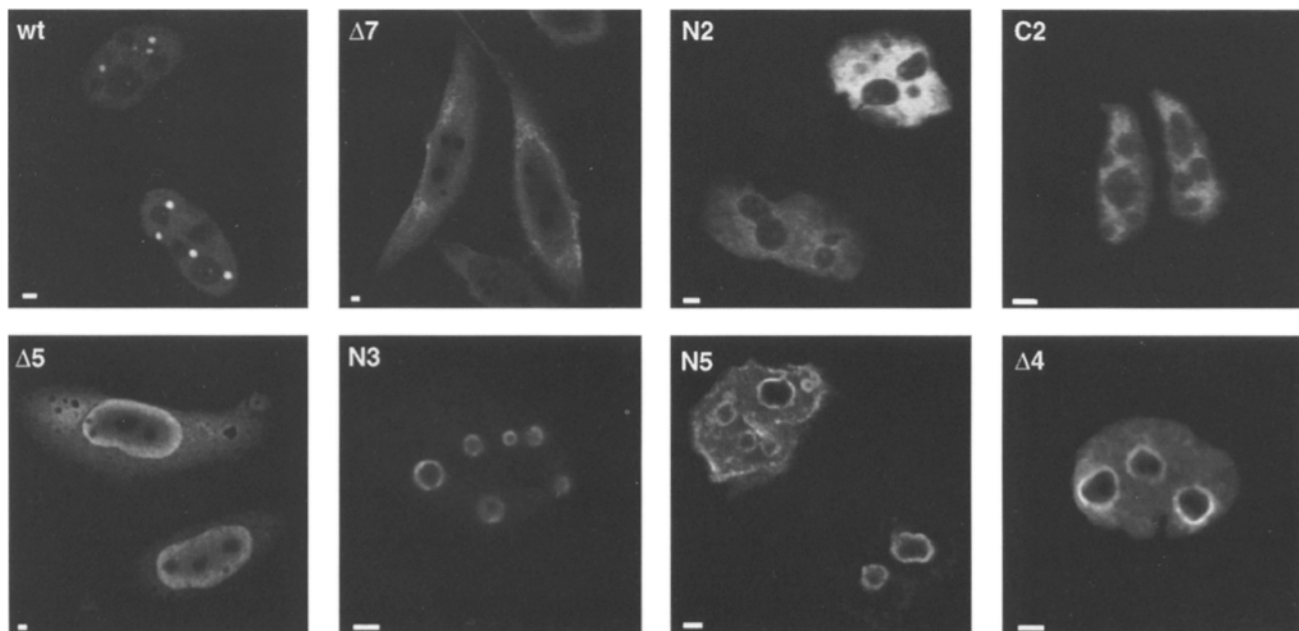

Figure 4. Transient expression of p80 coilin mutants in HeLa cells. Localization patterns of wild-type and mutant p80 coilin proteins were determined by immunofluorescence analysis in the confocal microscope using anti-myc antibodies on HeLa cells fixed 24–36 h after transient transfection with plasmids encoding the respective mutant constructs. Bar represents 2 μm.
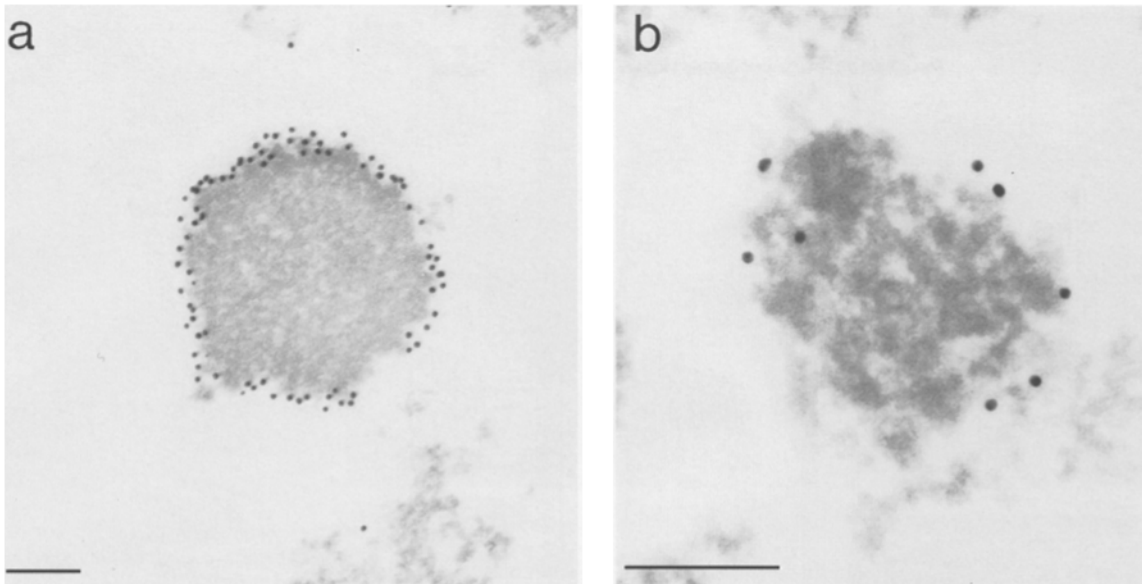
Figure 5. Immunoelectron microscopy of N3-transfected cells. (*a*) HeLa cells were transiently transfected with the plasmid encoding the mutant p80 coilin construct N3, fixed 30 h after transfection, immunolabeled with anti-myc antibodies pre-embedding and detected with secondary antibodies coupled to 15-nm gold particles using an intermediate layer (see Materials and Methods). Most of the immunogold label was found surrounding dense nucleoplasmic bodies which we term pseudo-coiled bodies. The absence of internal labeling in these structures is most likely due to the pre-embedding method employed. (*b*) Untransfected HeLa cells, immunolabeled with anti-p80 coilin antibodies pre-embedding and detected with secondary antibodies coupled to 15-nm gold particles without the use of an intermediate layer (see Materials and Methods). The immunogold label highly specifically concentrated on structures showing the typical coiled body morphology. Bar represents 0.2 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 foci, 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
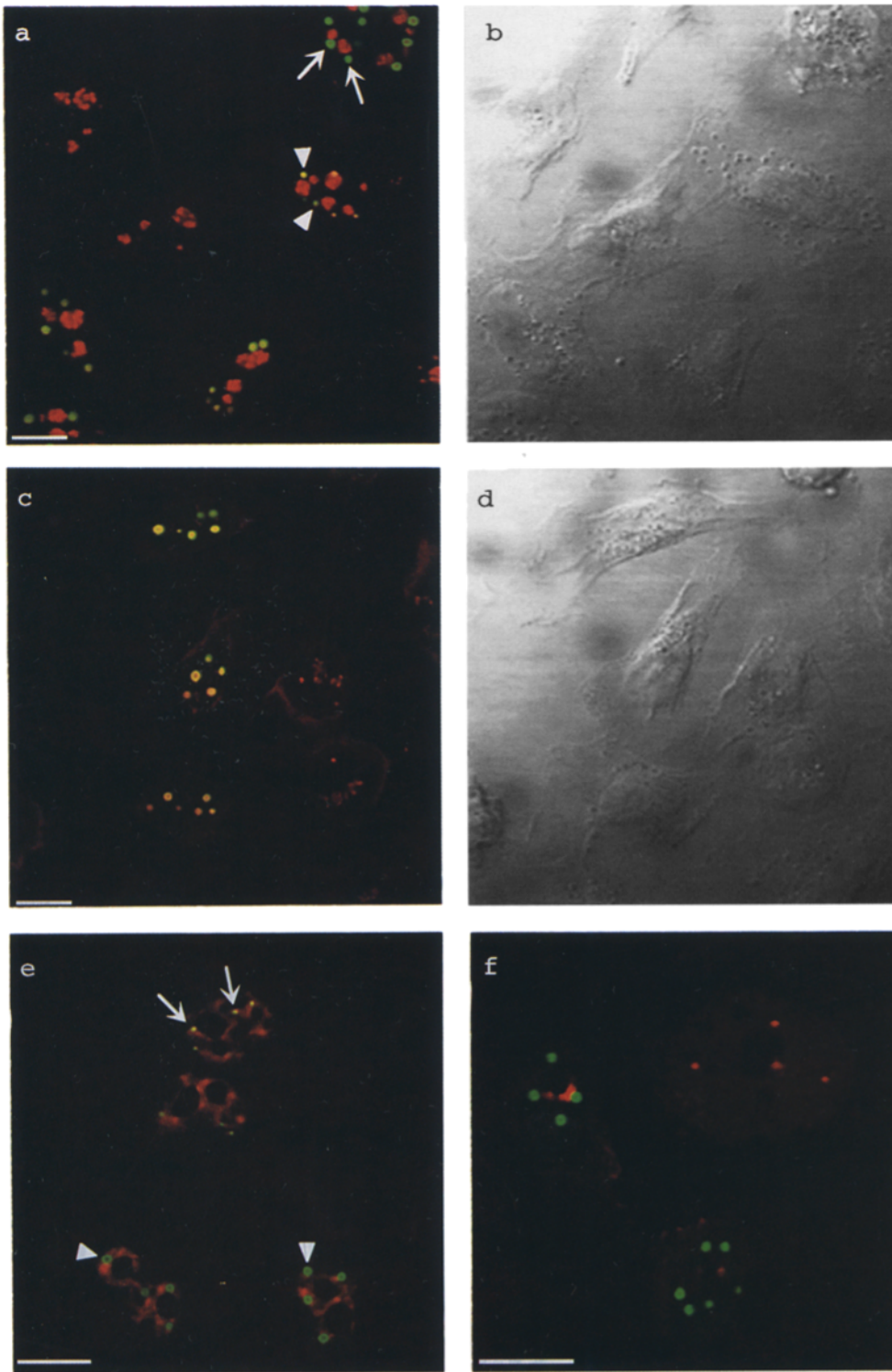
Figure 6. Mutant N3 forms pseudo-coiled bodies. HeLa cells were transiently transfected with the plasmid encoding p80 coilin mutant N3, fixed 24–36 h after transfection and analyzed by indirect immunofluorescence with the following combinations of antibodies: (*a*) anti-myc monoclonal antibody (*green*) and anti-fibrillarin autoantibodies (*red*), arrowheads show small foci 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-Nopp140 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 snRNPs in transfected cells. (*f*) Anti-myc monoclonal antibody (*green*) and anti-p80 coilin peptide rabbit antibody (*red*). Bar represents 10 μm.

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).

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 coilin 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 coilin was used to detect endogenous p80 coilin since this does not react with the N3 deletion mutant. We observe no colocalization of endogenous p80 coilin in N3 foci. Furthermore, in transfected cells the anti-p80 coilin antibody no longer labels prominent coiled bodies. In some cells p80 coilin 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 Nopp140 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 coilin. We will refer to these N3 containing foci as pseudo-coiled bodies.

Deletion Mutant Δ4 Promotes Redistribution of Fibrillarin in the Nucleolus

Several mutant derivatives of p80 coilin, including N4, N5, and Δ4, 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 Δ4 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 relocalization 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 Δ4 mutant protein. Little or no colocalization was observed between fibrillarin and the Δ4 protein. This experiment was repeated at different time points from 24 h to 60 h after transfection with the Δ4 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 Δ4 mutant still showed a significant level of nucleoplasmic staining (Fig. 7 a). At later times the Δ4 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 in-

terpretation of these data is that in the presence of the Δ4 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 Δ4 construct may prove lethal.

When the Δ4 mutant construct was microinjected into HeLa cells, rather than transfected, the redistribution of fibrillarin and accumulation of Δ4 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 Δ4 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 Δ4 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 Δ4 protein (Fig. 8, c–f). For this assay HeLa cells transiently transfected with the Δ4 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 Δ4 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 Δ4 protein inhibits RNA-polymerase I transcription but does not prevent nuclear transcription outside of nucleoli.

Mutant Δ4 Causes a Major Disruption of Nucleolar Architecture

The effect of expressing the Δ4 mutant protein on other nucleolar and nucleoplasmic antigens was also examined (Fig. 9). Double-labeling Δ4 transfected cells with anti-RNA polymerase I and anti-fibrillarin antibodies showed that expression of Δ4 protein results in a relocalization 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 Δ4 mutant protein (data not shown). This phenotype in transfected cells (Fig. 9 a, arrowhead) differs markedly from control cells where RNA-polymerase I is clearly localized within the nucleoli.
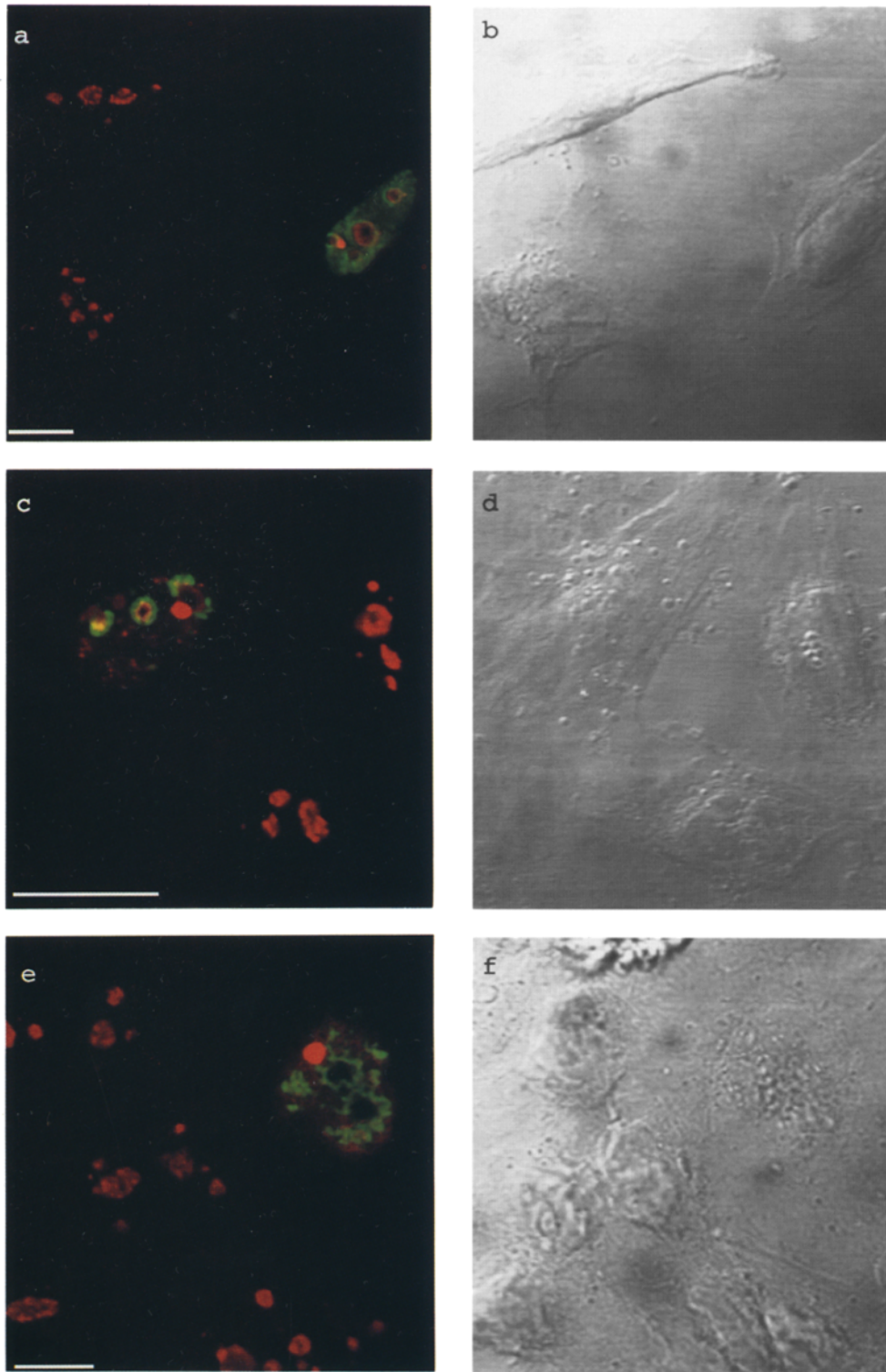
Figure 7. Mutant Δ4 promotes redistribution of fibrillarin. HeLa cells were transiently transfected with a plasmid encoding the p80 coilin mutant Δ4, fixed at varying times after transfection as indicated below and analyzed by immunofluorescence and Nomarski microscopy after staining with anti-myc monoclonal antibody (*green*) and anti-fibrillarin autoantiserum (*red*). Cells were fixed at following times after transfection: (*a* and *b*) 24 h; (*c* and *d*) 36 h; (*e* and *f*) 60 h. Bar represents 10 μm.

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. 9 *b* 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. 9 *b* 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. 9 *c*). In some transfected cells it also appeared that the level of cytoplas-
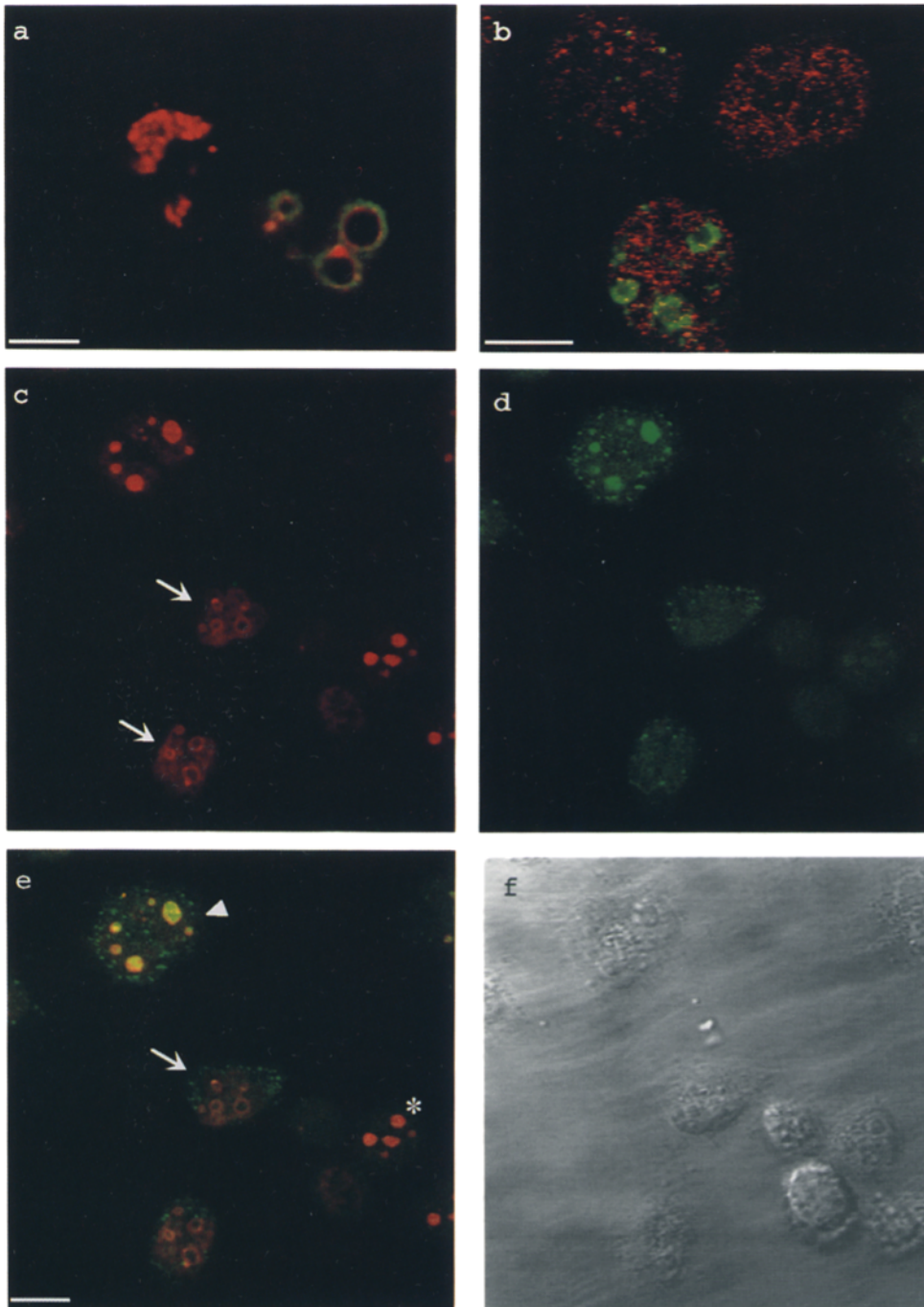
Figure 8. Effect of mutant Δ4 on transcriptional activity. (*a*) Cells were microinjected with the Δ4 mutant construct and fixed 4 h after injection. Cells were labeled with anti-fibrillarin autoantiserum (*red*) and monoclonal anti-myc antibody (*green*). (*b*) Cells were transfected with the Δ4 mutant 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 theΔ4 mutant construct and fixed 48 h after transfection. A subset of cells were micro-injected 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 anti-myc antibodies (data not shown).
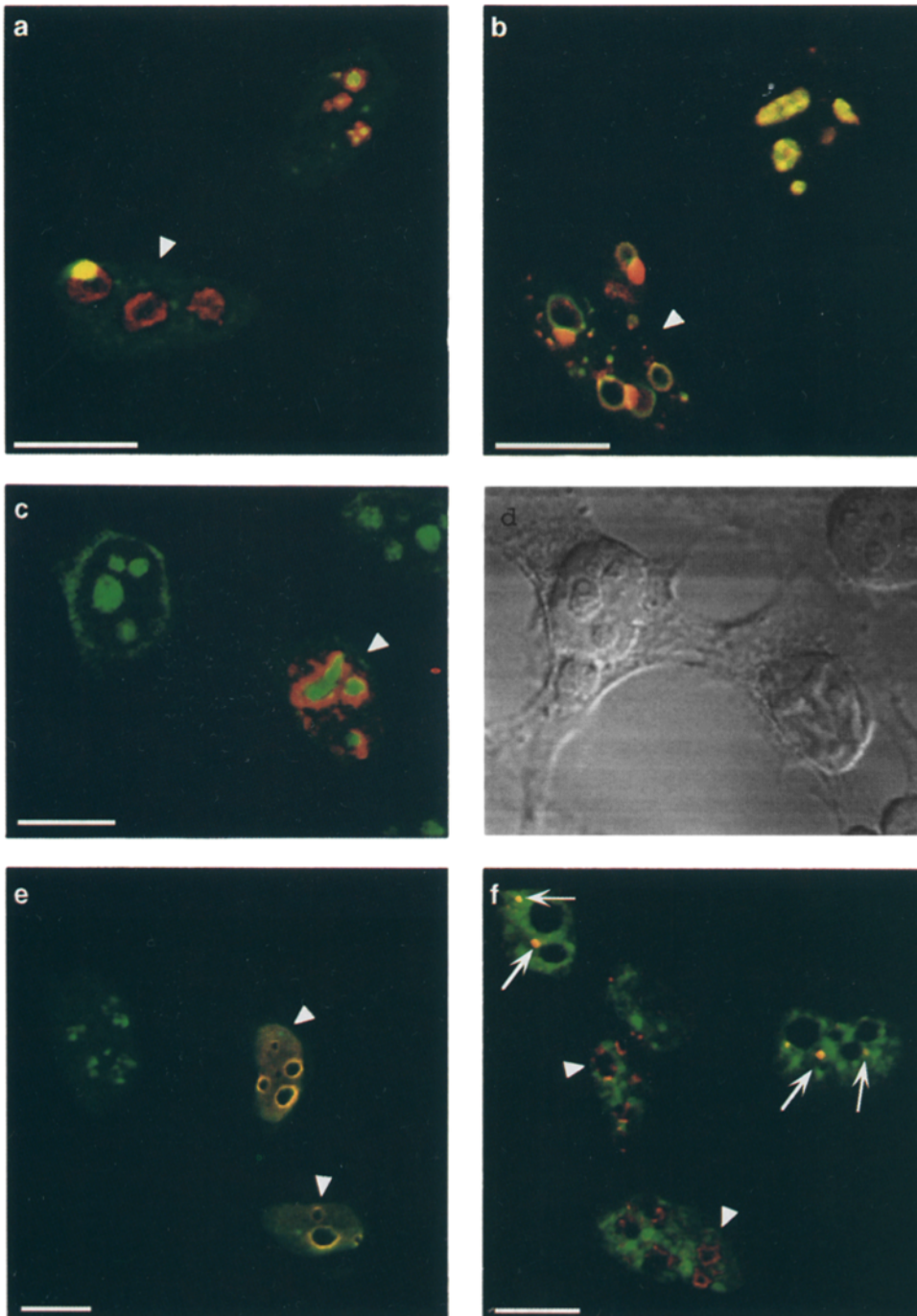
Figure 9. Mutant Δ4 promotes disruption of nucleolar architecture. HeLa cells were transiently transfected with the plasmid encoding the p80 coilin mutant Δ4, fixed 24–36 h after transfection and analyzed by immunofluorescence using the following antibodies or in situ probes: (*a*) anti-RNA polymerase I autoantiserum (*green*) and anti-fibrillarin monoclonal antibody (*red*), arrowhead indicates transfected cell. (*b*) Anti-nucleolin monoclonal antibody (*green*) and anti-fibrillarin autoantiserum (*red*), arrowhead indicates transfected cell. (*c* and *d*) Anti-myc monoclonal antibody (*red*) and anti-28S oligonucleotide hybridization probe (*green*). Arrowhead indicates transfected cell. *d* shows a confocal Nomarski image of the same group of cells analyzed by confocal fluorescence microscopy in *c*. (*e*) Anti-Nopp140 rabbit antibody (*green*) and anti-myc monoclonal antibody (*red*), arrowheads indicate transfected cells. (*f*) Anti-U2 snRNP B″ monoclonal antibody (*green*) and anti-p80 coilin rabbit antibody (*red*), arrows indicate coiled bodies in transfected cells, arrowheads indicate transfected cells. Bar indicates 10 μm.

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 immuno-

fluorescence 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
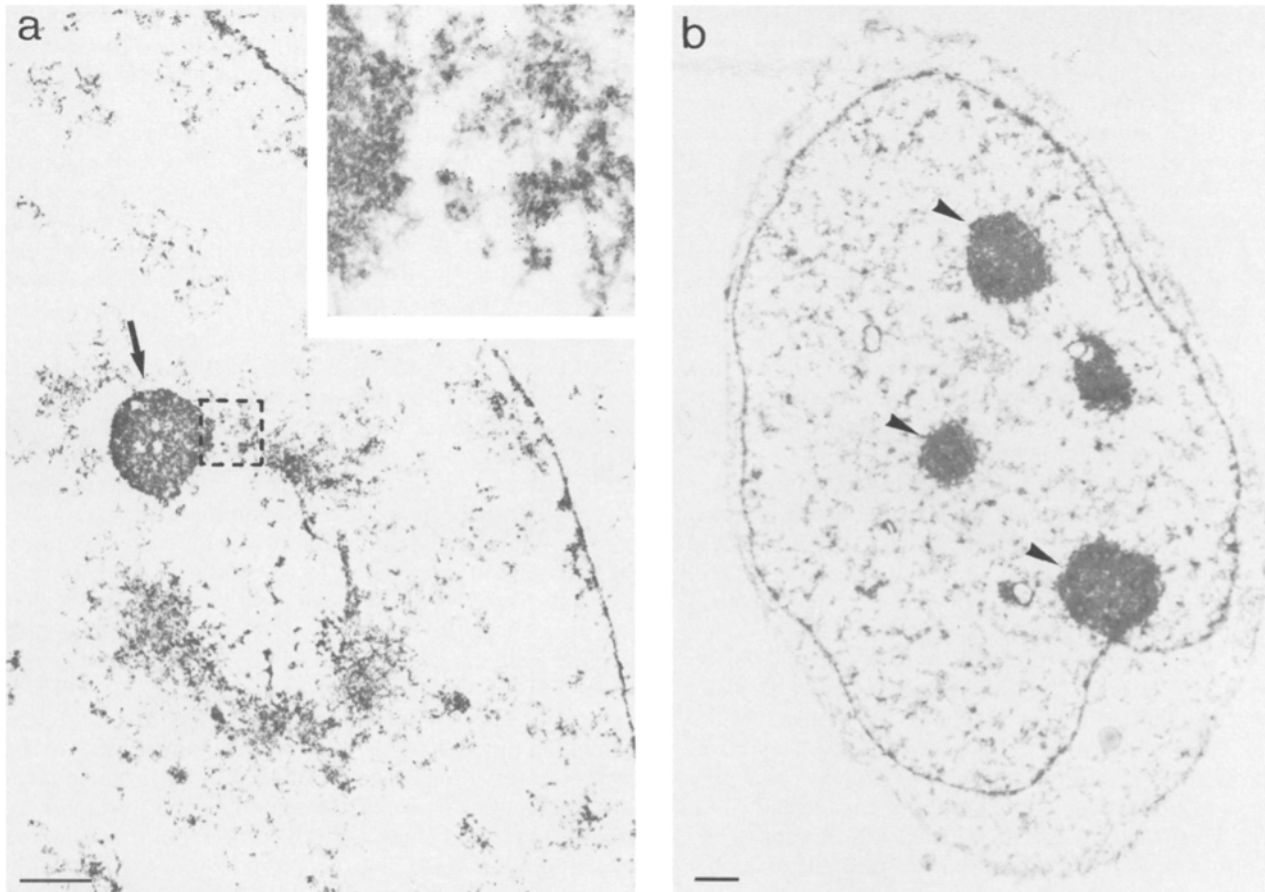
Figure 10. Immunoelectron microscopy of Δ4 transfected cells. (*a*) HeLa cells were transiently transfected with the plasmid encoding the mutant p80 coilin constructΔ4, fixed 30 h after transfection, immunolabeled with anti-myc antibodies pre-embedding and detected with secondary antibodies coupled to 5-nm gold particles. Most of the immunogold label was found concentrated over fibrillar structures encircling the remnant structures of the nucleoli. Inserts show enhanced magnification to make the immunogold label visible. The dense aggregate marked by an arrow is not labeled by anti-myc monoclonal antibodies. (*b*) Untransfected HeLa cells show the morphology of normal nucleoli (*arrowheads*) under the same extraction conditions. Bar represents 1 μm.

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 hSRPIα, 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 prevented localization in coiled bodies, even although 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 and Blobel, 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. 6 f). 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 and Blobel, 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.

References

Andrade, L. E., E. K. Chan, I. Raska, C. L. Peebles, G. Roos, and E. M. Tan. 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.

Ansorge, W., and R. Pepperkok. 1988. Performance of an automated system for capillary microinjection into living cells. *J. Biochem. Biophys. Methods.* 16:283–292.

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

Bohmann, K., J. Ferreira, N. Santama, K. Weis, and A. I. Lamond. 1995. Molecular analysis of the coiled body. *J. Cell Sci.* In press.

Brasch, K., and R. L. Ochs. 1992. Nuclear bodies (NBs): a newly "rediscovered"

organelle. *Exp. Cell Res.* 202:211–223.

Cajal, S. R. 1903. Un sencillo metodo de coloracion selectiva del reticulo proto-plasmatico y sus efectos en los diversos organos nerviosos de vertebrados e invertebrados. *Trab. Lab. Invest. Biol.* 2:129–221.

Carmo-Fonseca, M., K. Bohmann, M. T. Carvalho, and A. I. Lamond. 1994. P80 coilin. *In* Manual of Biological Markers of Disease. Vol. B8.2. W. J. v. Venrooij and R. N. Maini, editors. Kluwer Academic Publishers, 1–11.

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

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

Chan, E. K., S. Takano, L. E. Andrade, J. C. Hamel, and A. G. Matera. 1994. Structure, expression and chromosomal localization of human p80-coilin gene. *Nucleic Acids Res.* 22:4462–4469.

Dignam, J. D., R. M. Lebovitz, and R. G. Roeder. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* 11:1475–1489.

Dingwall, C., and R. A. Laskey. 1991. Nuclear targeting sequences—a consensus? [see comments]. *Trends Biochem Sci.* 16:478–481.

Dyck, J. A., G. G. Maul, W. J. Miller, J. D. Chen, A. Kakizuka, and R. M. Evans. 1994. A novel macromolecular structure is a target of the promyelocyte-retinoic acid receptor oncoprotein. *Cell.* 76:333–343.

Evan, G., G. Lewis, G. Ramsay, and M. Bishop. 1985. Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol. Cell. Biol.* 5:3610–3636.

Fakan, S., G. Leser, and T. E. Martin. 1984. Ultrastructural distribution of nuclear ribonucleoproteins as visualized by immunocytochemistry on thin sections. *J. Cell Biol.* 98:358–363.

Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132:6–13.

Fey, E. G., G. Krochmalnic, and S. Penman. 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.

Franke, W. W. 1988. Matthias Jacob Schleiden and the definition of the cell nucleus. *Eur. J. Cell Biol.* 47:145–156.

Fu, X. D., and T. Maniatis. 1990. Factor required for mammalian spliceosome assembly is localized to discrete regions in the nucleus. *Nature (Lond.).* 343: 437–441.

Gall, J. G. 1991. Spliceosomes and snurposomes. *Science (Wash. DC).* 252: 1499–1500.

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

Gerbi, S. A., R. Savino, B. Stebbins-Boaz, C. Jeppesen, and R. Rivera-Léon. 1990. A role for U3 small nucleolar ribonucleoprotein in the nucleolus? *In* The Ribosome- Structure, Function, and Evolution. W. E. Hill, A. Dahlberg, R. A. Garrett, P. B. Moore, D. Schlessinger, and J. R. Warner, editors. American Society for Microbiology, Washington, D.C. 452–469.

Green, S., I. Issemann, and E. Sheer. 1988. A versatile in vivo and in vitro eukaryotic expression vector for protein engineering. *Nucleic Acids Res.* 16: 369.

Habets, W. J., M. H. Hoet, J. B. De, d. K. A. Van, and V. W. Van. 1989. Mapping of B cell epitopes on small nuclear ribonucleoproteins that react with human autoantibodies as well as with experimentally-induced mouse monoclonal antibodies. *J. Immunol.* 143:2560–2566.

Henikoff, S. 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. *Gene (Amst.).* 28:351–359.

Jackson, D. A., A. B. Hassan, R. J. Errington, and P. R. Cook. 1993. Visualization of focal sites of transcription within human nuclei. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:1059–1065.

Jimenez, G. L., V. M. Segura, R. L. Ochs, L. I. Rothblum, R. Hannan, and D. L. Spector. 1994. Nucleologenesis: U3 snRNA-containing prenucleolar bodies move to sites of active pre-rRNA transcription after mitosis. *Mol. Biol. Cell.* 5:955–966.

Jordan, E. G. 1991. Interpreting nucleolar structure: where are the transcribing genes? *J. Cell Sci.* 98:437–442.

Koken, M. H. M., F. Puvion-Dutilleul, M. C. Guillemin, A. Viron, G. Linares-Cruz, N. Stuurman, L. de Jong, C. Szostecki, C. Chomienne, L. de Gos, et al. 1994. The t(15;17) translocation alters a nuclear body in a retinoic acid-reversible fashion. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:1073–1083.

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

Lamond, A. I., and B. S. Sproat. 1994. Isolation and characterization of ribonucleoprotein complexes. *In* RNA Processing–A Practical Approach, Vol. 1. S. J. Higgins and B. D. Hames, editors. Oxford University Press, Oxford. 103–140.

Langanger, G., J. de Mey, M. Moeremens, G. Daneels, M. de Brabender, and J. V. Small. 1984. Ultrastructural localization of α-actinin and filamin in cultured cells with the immunogold staining (IGS) method. *J. Cell Biol.* 99:1324–1334.

Li, H., and P. M. Bingham. 1991. Arginine/serine-rich domains of the su(wa) and tra RNA processing regulators target proteins to a subnuclear compartment implicated in splicing. *Cell.* 67:335–342.

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

Meier, U. T., and G. Blobel. 1990. A nuclear localization signal binding protein in the nucleolus. *J. Cell Biol.* 111:2235–2245.

Meier, U. T., and G. Blobel. 1992. Nopp140 shuttles on tracks between nucleolus and cytoplasm. *Cell.* 70:127–138.

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

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

Puvion, E. A., A. Viron, C. Assens, E. H. Leduc, and P. Jeanteur. 1984. Immunocytochemical identification of nuclear structures containing snRNPs in isolated rat liver cells. *J. Ultrastruct. Res.* 87:180–189.

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

Reimer, G., K. M. Pollard, C. A. Penning, R. L. Ochs, M. A. Lischwe, H. Busch, and E. M. Tan. 1987a. Monoclonal autoantibody from a (New Zealand black x New Zealand white)F1 mouse and some human scleroderma sera target an M_r 34,000 nucleolar protein of the U3 RNP particle. *Arthritis Rheum.* 30: 793–800.

Reimer, G., K. M. Rose, U. Scheer, and E. M. Tan. 1987b. Autoantibody to RNA polymerase I in scleroderma sera. *J. Clin. Invest.* 79:65–72.

Scheer, U., and D. Weisenberger. 1994. The nucleolus. *Curr. Opin. Cell Biol.* 6: 354–359.

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

Spector, D. L., X.-D. Fu, and T. Maniatis. 1991. Associations between distinct pre-mRNA splicing components and the cell nucleus. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:3467–3481.

Stelzer, E. H., I. Wacker, and J. De Mey. 1991. Confocal fluorescence microscopy in modern cell biology. *Semin. Cell Biol.* 2:145–152.

Tam, J. P. 1985. Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system. *PNAS USA.* 85:5409–5413.

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

Visa, N., D. F. Puvion, J. P. Bachellerie, and E. Puvion. 1993. Intranuclear distribution of U1 and U2 snRNAs visualized by high resolution in situ hybridization: revelation of a novel compartment containing U1 but not U2 snRNA in HeLa cells. *Eur. J. Cell Biol.* 60:308–321.

Wansink, D. G., W. Schul, I. van der Kraan, B. van Steensel, R. van Driel, and L. de Jong. 1993. Fluorescent labeling of nascent RNA reveals transcription by RNA polymerase II in domains scattered throughout the nucleus. *J. Cell Biol.* 122:283–293.

Weis, K., S. Rambaud, C. Lavau, J. Jansen, T. Carvalho, M. Carmo-Fonseca, A. Lamond, and A. Dejean. 1994. Retinoic acid regulates aberrant nuclear localization of PML-RAR α in acute promyelocytic leukemia cells. *Cell.* 76:345–356.

Weis, K., I. W. Mattaj, and A. I. Lamond. 1995. Identification of hSRP1a as a functional receptor for nuclear localization sequences. *Science (Wash. DC).* 268:1049–1053.

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