Association of snRNA genes with coiled bodies is mediated by nascent snRNA transcripts

Mark R. Frey*†, Arnold D. Bailey‡, Alan M. Weiner‡§ and A. Gregory Matera*†¥

Background: Coiled bodies are nuclear organelles that are highly enriched in small nuclear ribonucleoproteins (snRNPs) and certain basal transcription factors. Surprisingly, coiled bodies not only contain mature U snRNPs but also associate with specific chromosomal loci, including gene clusters that encode U snRNAs and histone messenger RNAs. The mechanism(s) by which coiled bodies associate with these genes is completely unknown.

Results: Using stable cell lines, we show that artificial tandem arrays of human U1 and U2 snRNA genes colocalize with coiled bodies and that the frequency of the colocalization depends directly on the transcriptional activity of the array. Association of the genes with coiled bodies was abolished when the artificial U2 arrays contained promoter mutations that prevent transcription or when RNA polymerase II transcription was globally inhibited by α-amanitin. Remarkably, the association was also abolished when the U2 snRNA coding regions were replaced by heterologous sequences.

Conclusions: The requirement for the U2 snRNA coding region indicates that association of snRNA genes with coiled bodies is mediated by the nascent U2 RNA itself, not by DNA or DNA-bound proteins. Our data provide the first evidence that association of genes with a nuclear organelle can be directed by an RNA and suggest an autogenous feedback regulation model.

Addresses: *Department of Genetics and †Program in Cell Biology, Case Western Reserve University and University Hospitals of Cleveland, Cleveland, Ohio 44106-4955, USA. ‡Department of Molecular Biophysics & Biochemistry and §Department of Genetics, Yale University, New Haven, Connecticut 06520-8114, USA. ¥Center for Human Genetics, Case Western Reserve University and University Hospitals of Cleveland, Cleveland, Ohio 44106-4955, USA.

Correspondence: A. Gregory Matera
E-mail: gxm26@po.cwru.edu

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Background

Coiled bodies are nuclear organelles that range in size from 0.1 to 1.0 microns and can often be seen in the light microscope. Coiled bodies were discovered around the turn of the century [1] but their functions have remained mysterious despite growing knowledge about the composition, localization and formation of the organelle. Although originally defined morphologically, coiled bodies are usually identified by the presence of the signature protein p80 coilin and have been found to contain an extraordinarily wide range of nuclear components. These components include U small nuclear ribonucleoproteins (snRNPs; both major and minor classes) involved in messenger RNA (mRNA) splicing [2–4], U7 snRNP required for histone mRNA 3′ processing [5,6], and U3 small nucleolar (sno)RNP which participates in nucleolar ribosomal RNA (rRNA) processing [7]. Coiled bodies also contain nucleolar proteins such as fibrillarin, Nopp140, NAP57 and topoisomerase I [8–10], in addition to a number of other cellular components belonging to the basal transcription and cell cycle control machinery [11–13].

Although the composition of coiled bodies is relatively well understood, the organelles themselves are surprisingly heterogeneous in number, size and morphology [14,15]. Some cell lines such as HT1080 have a discrete number of similarly sized coiled bodies (see Results), whereas others have multiple coiled bodies of irregular size [16] or no detectable coiled bodies at all [17]. No equivalent structure has been found in budding yeast, but a good case can still be made that coiled bodies are a universal component of metazoan cells [15]. It is not clear what ‘glues’ the diverse components of coiled bodies together into a single distinct organelle; coiled-body-like structures can assemble in the absence of p80 coilin but are devoid of the Sm class of snRNP core proteins [18].

Coiled bodies are dynamic organelles, disassembling in mitosis and reassembling in mid G1 phase after nucleologenesis and the resumption of transcription [14,19,20]. Additional evidence for the dynamic nature of these organelles comes from the observations that snRNPs no longer accumulate within coiled bodies when transcription is shut off by α-amanitin [2] or during terminal differentiation [21]. There may also be a finite number of snRNP-binding sites within coiled bodies; microinjected U7 snRNA transcripts replace endogenous U7 in the coiled bodies of *Xenopus* oocytes [22]. This apparent flux of materials through coiled bodies suggests a role for these structures in snRNA metabolism.

The snRNAs are transcribed in the nucleoplasm, but snRNAs of the Sm class (including U1, U2 and U7 snRNA) are matured in the cytoplasm. Following export, these

RNAs undergo assembly with Sm proteins, cap trimethylation, and 3′ processing before being imported back into the nucleus [23]. At steady state, mature spliceosomal snRNPs including U1 and U2 localize diffusely throughout the nucleoplasm (corresponding to perichromatin fibrils) but also to speckled domains (interchromatin granules) and coiled bodies (bright foci), as described in recent reviews [24–26]. Nucleoplasmic U snRNPs are presumably functional in mRNA splicing [24,27], whereas speckles might serve as storage depots for inactive, hyperphosphorylated forms of the mature U snRNPs and RNA polymerase II [28–30]. The function of the U snRNPs in coiled bodies is unknown, as are the forces that govern partitioning of U snRNPs into these three nuclear compartments.

Two important clues suggest that coiled bodies might play a role in snRNP and/or snoRNP biogenesis. First, coiled bodies preferentially associate with specific chromosomal loci in vertebrates. These loci include the replicative histone gene clusters in amphibia [31,32] and mammals ([6]; M.R.F. and A.G.M., unpublished observations), the tandemly repeated human U1 and U2 snRNA genes [6,33], and the human U3 snoRNA genes [34]. The association of U1, U2 and U3 genes with a nuclear organelle that contains mature U1 and U2 snRNPs, and U3 snoRNP, suggests that there is a feedback circuit: a fraction of the mature snRNPs and snoRNPs might return to the snRNA genes themselves to regulate snRNA synthesis and/or U snRNP assembly [6,26]. The association of replicative histone genes with coiled bodies containing mature U7 snRNP also suggests there is feedback circuit: mature histone mRNA levels might be regulated by the availability of U7 snRNP in the coiled body. Indeed, as U7 snRNP associates with the 3′ terminal stem–loop of the histone mRNA precursors, histone genes might associate with the coiled body through the nascent histone mRNA.

A second clue suggesting that coiled bodies participate in snRNP and/or snoRNP biogenesis is that coiled bodies colocalize with twin structures known as 'gemini of coiled bodies' or gems [35], which play a role in the snRNP life cycle [36,37]. The 'survival motor neurons' gene product, SMN, is distributed throughout the cytoplasm, but nuclear staining is restricted to coiled bodies [35,38]. Deletion of the human *SMN1* gene leads to the autosomal recessive disorder called spinal muscular atrophy (SMA). Surprisingly, the defect in this disease appears to be one of U snRNP assembly/re-assembly [37,39] although this interpretation has been disputed [40]. Thus, SMN participates in cytoplasmic snRNP assembly and is thought to accompany the assembled snRNPs on their journey to nuclear gems and coiled bodies. The colocalization of the SMN complex in coiled bodies suggests that these structures participate in the snRNP life cycle. For example, excess snRNPs could accumulate in coiled bodies, or coiled bodies could be an obligate waystation on the U snRNP biogenesis pathway.

To explore the possible role of coiled bodies in the regulation of snRNA synthesis and snRNP biogenesis, we constructed stable human cell lines containing artificial tandem arrays of human U1 and U2 genes. We find that the artificial arrays, like the endogenous U1 and U2 loci *RNU1* and *RNU2*, associate with coiled bodies and that the frequency of association is directly proportional to the transcriptional activity of the artificial array. This association is abolished when the U2 coding region is replaced by random sequences of comparable length. We conclude that the association between U2 genes and coiled bodies is mediated by nascent U2 snRNA, and we speculate regarding the mechanism and function of this association.

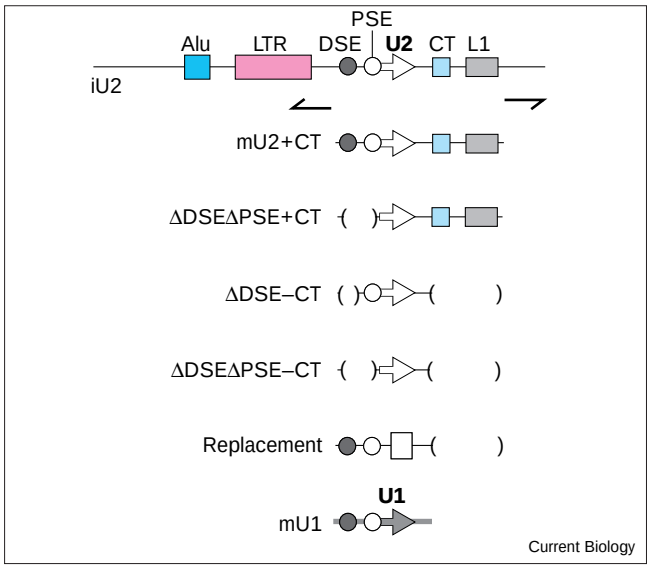
Results

Artificial arrays of U1 and U2 snRNA genes associate with coiled bodies

Coiled bodies in human cell lines often appear to colocalize with the gene clusters encoding histones, U1 and U2 snRNAs, and U3 small nucleolar RNA [6,33,34]. The fluorescent signals corresponding to the coiled bodies and the genes seldom overlap completely, however, indicating that the coiled body and the genes could in fact be as much as 50 kb apart [41]. In order to demonstrate that coiled bodies associate specifically with snRNA genes and not with adjacent chromosomal sequences, we took advantage of human cell lines containing artificial tandem arrays of U1 or U2 snRNA genes that were originally constructed [42,43] to study adenovirus-12-induced chromosome fragility [42–46]. Each artificial U1 or U2 locus contains a perfect head-to-tail tandem array of a wild-type or mutant U1 or U2 repeat unit (Figure 1). We found that the artificial U2 tandem arrays also colocalized with coiled bodies, and that the U2 and coiled body signals often overlapped, as observed for the endogenous *RNU2* loci (Figure 2). The tetraploid A37 cell line was especially revealing. A37 contains two to eight coiled bodies, four endogenous *RNU2* loci and one artificial U2 locus with 48 tandem copies of the natural 6.1 kb U2 repeat unit [42]. Multiple associations of U2 loci with coiled bodies were common in A37 cells, and in rare cases all four endogenous *RNU2* loci and the large artificial U2 array colocalized with coiled bodies (Figure 2b). Note that the large artificial U2 array was easily distinguished from the endogenous *RNU2* loci that each contained only 9–13 gene copies [47]. Thus, partial overlap between the U2 and p80 coilin signals indicates that coiled bodies might interact with only a portion of each array, or localize to the general vicinity of the array by unknown mechanisms. In either case, the data clearly demonstrate that U2 genes are sufficient for coiled body colocalization.

The frequency with which the artificial U2 array associated with coiled bodies correlated well with the relative transcriptional activity of the array (Figure 3). All the U2 genes in each artificial array were marked by a functionally silent point mutation in the snRNA coding region (U87C),

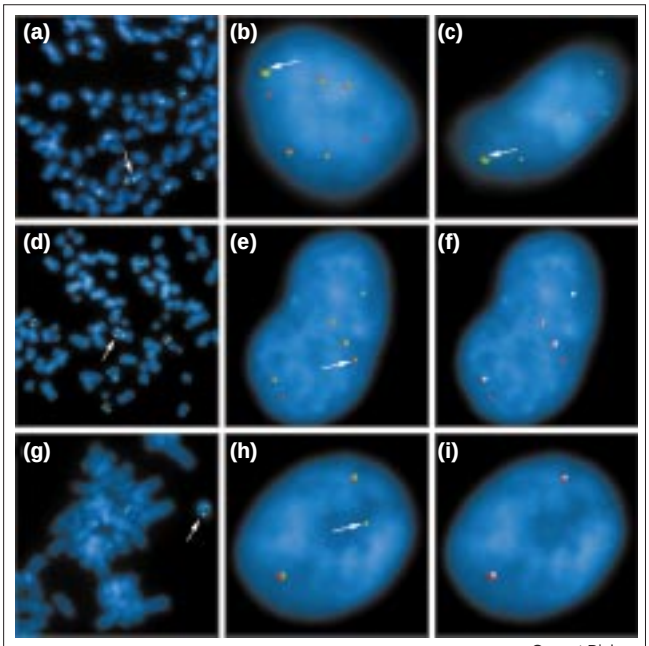
Figure 1



Constructs used to build artificial tandem arrays of human U1 and U2 genes. The natural 6.1 kb repeat unit of the *RNU2* locus (intact U2; iU2) is shown on top; indicated below is the extent of each subclone that was multimerized to generate an artificial tandem array. DSE and PSE are distal and proximal sequence elements, respectively, and are the equivalent of the enhancer and promoter sequences for this special class of TATA-less snRNA promoters. The solo long terminal repeat (LTR) belongs to the HERV-K10 family; CT is a $(CT)_{\sim 70}$ microsatellite; and L1 is a 3′ terminal fragment of a LINE-1 element [47,74]. Arrows beneath the iU2 construct mark the locations of PCR primers used to generate fluorescence *in situ* hybridization (FISH) probes (see Materials and methods). Deletions relative to the iU2 repeat are marked by parentheses. Replacement of the U2 coding region with a heterologous sequence is indicated by a white box. The U1 minigene construct (mU1) [43] is shown at the bottom.

enabling us to use a simple primer extension assay [42] to determine the steady state ratio of mature U2 snRNA contributed by the artificial and natural U2 arrays. Remarkably, a plot of the RNA ratio versus the frequency of coiled body association revealed a linear correlation between the relative transcriptional activity of the array and the frequency of colocalization with coiled bodies (Figure 3). The behavior of the artificial U2 array in the A41 cell line is illustrative (Figure 2c). The A41 line, like A37 (Figure 2b), has five U2 loci — four natural *RNU2* loci and one artificial U2 tandem array. Although out-numbered four to one, the artificial U2 array in A41 is strongly transcribed and accounts for more than 70% (2.5:1 or 2.5 out of 3.5, Figure 3a) of steady state U2 snRNA levels. Correspondingly, the artificial U2 array in A41 accounts for nearly half (18 out of 38) of the observed U2 gene–coiled body associations (Figure 3a). In contrast, the artificial U2 tandem array in A40 has nearly the same number of U2 repeats as in A41, but is not as highly transcribed and does not associate as frequently with coiled bodies. Thus, the more transcriptionally active the U2 locus, the more often it associates with a coiled body.

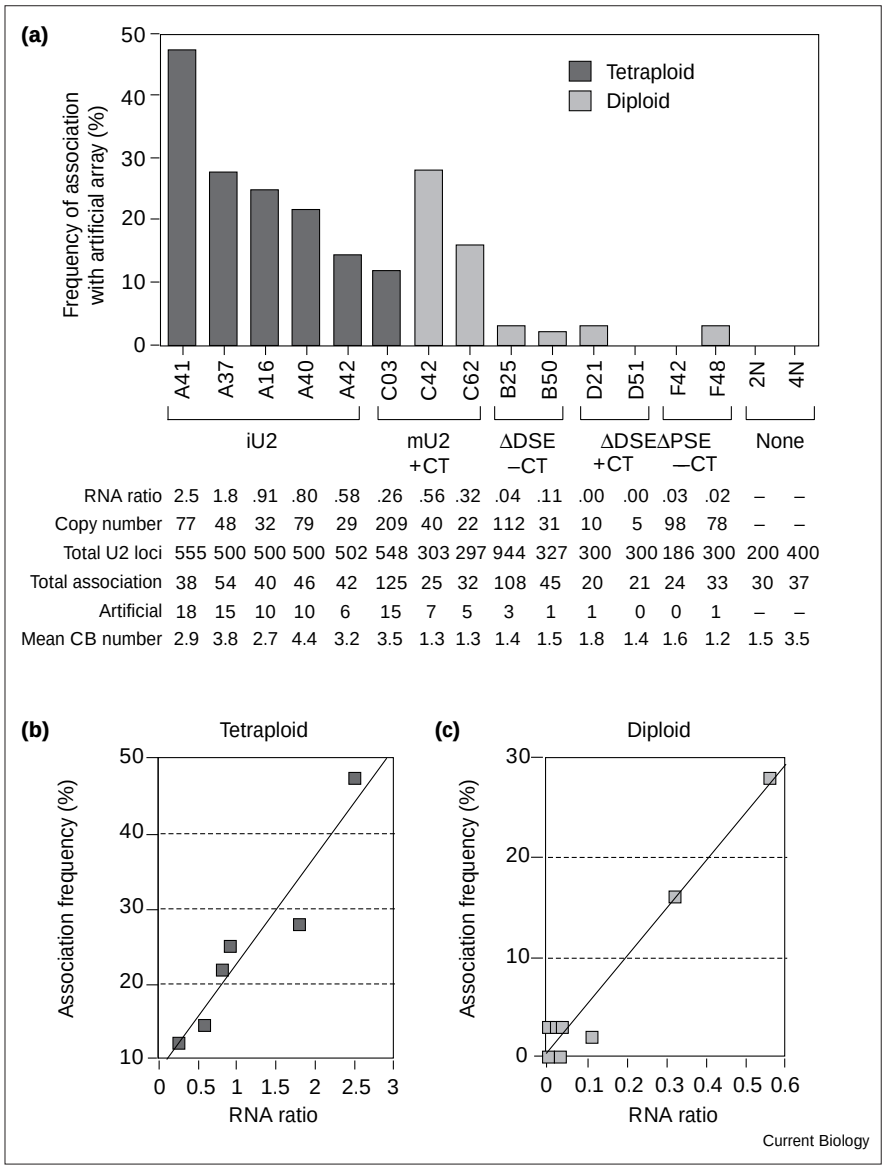
Figure 2



Colocalization of coiled bodies with the artificial U2 and endogenous *RNU2* arrays. Only representative cell lines are shown; the full data-set is presented in Figure 3. Cell lines are described in Figure 3a and contain stably integrated tandem arrays of the normal, deleted, or mutated U2 constructs shown in Figure 1. Throughout the figure, the artificial and endogenous U2 arrays are shown in green, coiled bodies in red, and the endogenous *RNU2* loci in white. **(a)** Metaphase FISH using tetraploid cell line A37 shows the locations of the four endogenous U2 signals as well as the artificial arrays (arrow). A fraction of these cells displayed homogeneously stained regions, presumably resulting from duplications of the chromosome bearing the iU2 construct; see Yu *et al.* [75] for details. **(b)** Interphase FISH reveals that the artificial U2 genes (arrow) associate with coiled bodies. All five of the U2 loci in this particular cell are associated with coiled bodies. Note that despite separation by a large metaphase distance, the two artificial U2 gene clusters on the endoreduplicated A37 chromosome colocalize in interphase. **(c)** A typical interphase association of an artificial array with a coiled body in A41 cells. Note that the FISH signals from the artificial arrays (arrow) in cells bearing the iU2 constructs are much larger than those of their endogenous counterparts. **(d–f)** Transcriptionally active U2 minigene constructs associate with coiled bodies. (d) Metaphase spread of cell line C03; an arrow denotes the location of the artificial array. (e,f) The FISH signals from the smaller, minigene constructs were often indistinguishable from those of the endogenous arrays, so a differentially labeled (Cy5) PCR probe was used to mark the locations of the endogenous sequences – white signals in (f). For each of the cell lines shown in Figure 3, > 100 cells were scored and the number of U2 loci (both wild type and artificial) that associated with coiled bodies was counted. An example of an artificial U2 array colocalizing with a coiled body is shown – arrow in (e). **(g–i)** Examples of (g) metaphase and (h,i) interphase F42 cells, which contain artificial arrays of transcriptionally inactive U2 genes (marked by arrows). Note that whereas the artificial loci – arrow in (h) – fail to associate with coiled bodies, the endogenous loci – white in (i) – serve as internal positive controls.

The coiled bodies that associate with artificial U2 arrays contain typical coiled body components. Cell lines A37 and

Figure 3



Association of artificial U2 tandem arrays with coiled bodies correlates with the relative transcriptional activity of the array. (a) Percentage association with the artificial arrays was determined by scoring the total number of U2 loci (artificial and endogenous) that colocalized with coiled bodies and then calculating the fraction of these that associated with the artificial arrays. The ratio of marked to unmarked U2 snRNA, derived from the artificial and endogenous U2 snRNA genes, respectively, was determined by primer extension and remained stable over many cell passages [42,48]. Mean coiled body (CB) number was determined by examining > 100 cells per line. Data for tetraploid and diploid HT1080 derivatives are shown separately. As shown in the table in (a), the parental diploid HT1080 cells (labeled 2N) have an average of 1.5 coiled bodies per cell, whereas tetraploid subclones (4N) average 3.5 coiled bodies. These data are also reflected in the mean number of coiled bodies detected in cells bearing artificial U2 constructs. Interestingly, although the number of coiled bodies per cell doubled upon tetraploidization, the ratio of U1 to U2 snRNA remained constant (see Materials and methods) and the basal frequency of *RNU2*–coiled body association did not change. Approximately 35% of both diploid and tetraploid HT1080 cells (but only 15% and 9%, respectively, of all the *RNU2* loci) showed at least one coiled body association. Thus, the number of coiled bodies appears to be proportional to the number of chromosomes, but the frequency of coiled body colocalization with U2 genes does not. (b,c) Data in (a) replotted. The tetraploid data can be fitted by $y = 0.144x + 0.083$, $r = 0.954$; the diploid data by $y = 0.484x + 0.003$, $r = 0.980$.

A41, containing tandem arrays of intact U2 (iU2) repeat units (Figures 1,3), were probed with biotinylated antisense 2′-OMe U2 or U7 oligonucleotides. Following incubation and detection with anti-p80-coilin antibodies, the cells were fixed, denatured, and then hybridized with a U2 gene probe as in Figure 2. Fluorescence *in situ* hybridization (FISH) signals corresponding to the artificial iU2 array and endogenous *RNU2* loci were easily distinguished morphologically. In addition to p80 coilin, coiled bodies that associated with the artificial arrays were found to contain U2 and U7 snRNAs, and the U2B″ protein, as well as the nucleolar proteins fibrillarin and Nopp140 (data not shown).

We were able to generalize our results to other snRNA genes by assaying cell lines mU1-96 and mU1-99, which contain tandem arrays of a transcriptionally active U1 minigene construct [43]. Although the natural U1 genes (*RNU1*) in HT1080 cells associate with coiled bodies at a lower frequency than the corresponding *RNU2* loci, a significant fraction of the artificial U1 arrays associated with coiled bodies (data not shown, but see later). These data provide additional evidence that the association of snRNA genes with coiled bodies is likely to be functionally significant.

Promoter deletion or inhibition of transcription abolishes association of U2 genes with coiled bodies

To identify functional elements within the U2 repeat unit that are necessary for association with coiled bodies, we analyzed cell lines containing mutant U2 arrays (Figure 1). As revealed by our minigene constructs, most of the

natural 6.1 kb U2 repeat unit was dispensable for association with coiled bodies. Tandem arrays of a 1.7 kb U2 minigene construct (mU2+CT) that includes the downstream $(CT)_n$ microsatellite were sufficient for colocalization (Figure 2). Arrays of U2 minigenes lacking the U2 enhancer (ΔDSE), or lacking both the enhancer and promoter (ΔDSEΔPSE), did not preferentially associate with coiled bodies (Figures 2,3), suggesting that active transcription is required for colocalization. Although we were unable to recover large tandem arrays of transcriptionally active U2 minigenes lacking the downstream $(CT)_n$ microsatellite [48], artificial arrays of U1 minigenes lacking the equivalent $(CT)_n$ microsatellite were readily recovered [43] and these colocalized with coiled bodies. Therefore, the CT microsatellite is unlikely to play any role in coiled body association; U1 minigenes lacking this element colocalize with coiled bodies, and promoter-deleted mU2+CT constructs do not. Thus, coiled bodies associate with ectopically expressed U1 and U2 snRNA genes but not with transcriptionally impaired mutants.

Consistent with these observations, global inhibition of RNA polymerase II transcription by α-amanitin or actinomycin D completely disrupted the coiled body–*RNU2* association. Carmo-Fonseca *et al.* [2] originally showed that localization of U snRNPs in coiled bodies was dependent upon transcription and that inhibition of RNA polymerase II causes splicing factors to undergo a dramatic reorganization from the characteristic speckled pattern into large clusters. These clusters were later shown to correspond to interchromatin granules and were also observed upon inhibition of pre-mRNA splicing [49]. We also find that p80 coilin staining remains punctate for some time after α-amanitin treatment, although coilin eventually accumulates around the nucleolus, forming a cap-like structure ([2]; data not shown). We never observed colocalization of U2 genes with coilin foci in cells treated with α-amanitin, however (5 μg/ml for 5 hours; see Supplementary material published with this paper on the internet). Similar results were obtained with actinomycin D (5 μg/ml for 1–3 hours; data not shown). As a control, the U2B″ protein (a marker for U2 snRNPs) was found to relocalize from speckles to large clusters in treated cells ([2]; see Supplementary material).

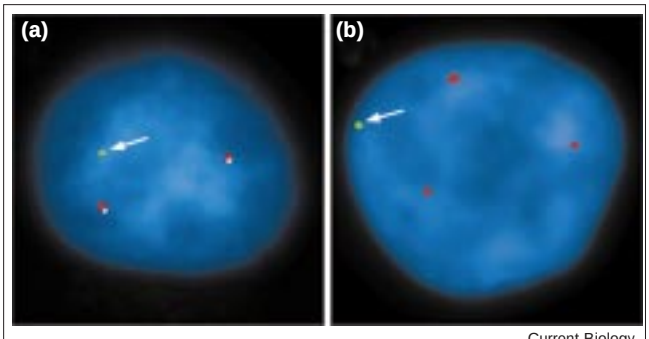
Association of U2 genes with coiled bodies requires transcription of U2 snRNA coding sequences

The data in Figures 2 and 3 suggest that U1 and U2 snRNA transcriptional control signals might be necessary, and perhaps sufficient, for association of coiled bodies with snRNA genes. To test this hypothesis, we generated artificial tandem arrays of a hybrid construct in which U snRNA transcriptional control elements (a U2 enhancer/promoter and a U1 3′ end-formation signal) drive expression of a heterologous 170 bp 'replacement' sequence derived from the first intron of the adenovirus 2 major late transcription unit (Figure 1). Surprisingly, these artificial arrays, in which all but the first 13 nucleotides of the U2 snRNA coding region had been replaced by heterologous sequence, did not colocalize with coiled bodies (Figure 4). Failure of the arrays to associate with coiled bodies was not due to a lack of transcription, as the transcripts were easily detectable by RNA FISH (Figure 4). Two independent cell lines harboring arrays of the replacement construct (U2Ad-84 and U2Ad-173) were assayed; colocalization of the RNA FISH signals with coiled bodies was never observed in either cell line (Figure 4, > 100 cells scored). As expected, sequential hybridization for replacement RNA and DNA, using differentially labeled probes, identified the same sites, and treatment with RNase A abolished the RNA FISH signal (data not shown). Thus, following transcription, the replacement RNA is presumably degraded and apparently does not accumulate at any other sites in the nucleus. Taken together, these results demonstrate that U2 promoter and terminator sequences are not sufficient, and might not even be necessary, for the coiled body association. Any strict requirement for a U snRNA promoter would be difficult to test rigorously because a U snRNA promoter is required for recognition of a U snRNA 3′ end-formation signal [50,51]. We conclude that nascent (or possibly newly transcribed) U2 snRNA is required for association of coiled bodies with U2 genes.

Coiled bodies can simultaneously associate with multiple chromosomal loci

Given the relatively small number of coiled bodies (1.5 ± 0.7 per cell for diploid HT1080, Figure 3a) and the large number of coiled-body-associated loci, it seemed likely that several chromosomal loci might associate with a

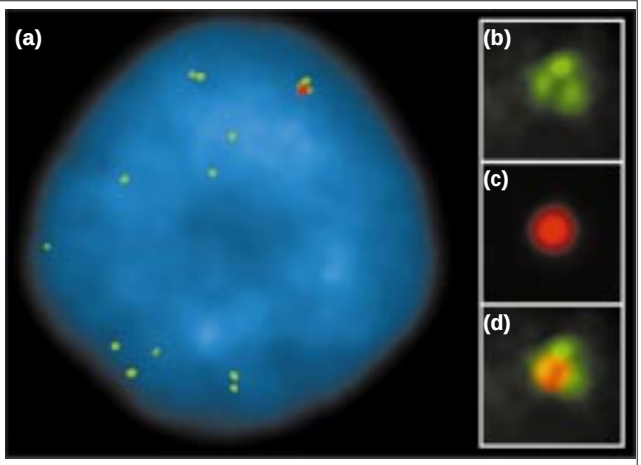
Figure 4



'Replacement' arrays do not colocalize with coiled bodies. Cells were counterstained with DAPI (blue), and coilin immunofluorescence is shown in red. (a) DNA hybridization with differentially labeled probes to the replacement array (green) and endogenous *RNU2* loci (white) reveals that the exogenous constructs do not associate with coiled bodies. The arrow marks the location of the replacement array. (b) Hybridization to nascent replacement RNA (green, arrow) demonstrates that the replacement constructs are transcribed, yet they do not associate with coiled bodies.

single coiled body [33,34]. We tested this idea using a mixed probe corresponding to seven different loci. Five of these loci (*RNU1*, *RNU2*, *RNU3*, *HIST1* and *HIST2*) have already been shown to preferentially associate with coiled bodies [34]. The other two clones correspond to *RNU7* and *RNU12* loci and also show a statistically significant association with coiled bodies relative to control gene probes (E. Jacobs, M.R.F. and A.G.M., unpublished observations). FISH was performed using all seven probes labeled with biotin and detected with fluorescein isothiocyanate (FITC)–avidin; p80 coilin was detected by immunofluorescence as in Figure 2. We examined diploid HT1080 cells and scored only those cells with 14 visible FISH signals. Most of the loci in any given cell were not associated with coiled bodies (Figure 5). Given the known territorial constraints of the chromosomes within each interphase nucleus [25], the small number of coiled bodies per cell (~1.5), and the wide distribution of the loci examined, these results were not unexpected. By scanning the slide, however, we found that subsets of the seven loci readily associated with coiled bodies and that the mixed probe occasionally generated a visible rosette surrounding a single coiled body (Figure 5d). Thus, coiled bodies can simultaneously interact with multiple genetic loci and perhaps coordinately regulate the expression of these loci (see later).

Figure 5



Coiled bodies can simultaneously interact with multiple DNA loci. Diploid parental HT1080 cells were hybridized with seven different probes, corresponding to the *RNU1*, *RNU2*, *RNU3*, *RNU7*, *RNU12*, *HIST1* and *HIST2* loci. Fourteen hybridization signals (green) are clearly detected, three of which are in direct contact with a coiled body (red). **(a)** The three different source images (red, green and blue) were merged using a 'maximum pixel' algorithm that best illustrates the positional information. **(b–d)** The individual source images from the region surrounding the coiled body: (b) the unmerged FISH signal (green) which clearly shows three of the fourteen loci; (c) the coiled body (red); and (d) the merged image. In (d), a 'color blending' algorithm was used to display the rosette configuration; the overlap between the red and green fluorescence signals results in a yellow color.

Discussion

We have shown that artificial arrays of U1 and U2 snRNA genes colocalize with coiled bodies, thus demonstrating that the snRNA genes themselves, and not flanking chromosomal sequences, suffice for colocalization. We also show that only transcriptionally competent U2 genes associate with coiled bodies, and that the frequency of association correlates directly with the relative transcriptional activity of the artificial U2 arrays. Most importantly, we find that the association of artificial U2 arrays with coiled bodies is abolished when the U2 snRNA coding region is replaced by a random sequence of comparable length. We conclude that coiled bodies interact (directly or indirectly) with the nascent U2 snRNA transcripts. Although the resolution of the light microscope cannot provide conclusive proof of a physical interaction, our results demonstrate that nascent U2 snRNA is required for the association of U2 genes with coiled bodies.

What is the glue that tethers U2 snRNA genes in the vicinity of a coiled body? One attractive hypothesis is that transcription of snRNA (and perhaps histone) gene clusters nucleates coiled bodies, in the same way as transcription of rDNA (the 'nucleolus organizer region') creates nucleoli [52]. This seems unlikely for two reasons, however. First, although often associated with snRNA genes, coiled bodies do not accumulate newly synthesized RNA [12,13,32,53]. Second, if snRNA transcription were able to nucleate formation of coiled bodies, one might expect many or all coiled bodies to be associated with snRNA gene clusters. In fact, coiled-body-like structures can form in artificial nuclei assembled from *Xenopus* egg extract using bacteriophage lambda as the DNA source [54,55]. Thus, at least in some circumstances, coiled bodies can form in the complete absence of cognate genomic DNA or authentic transcription units.

An alternative hypothesis is that coiled bodies may assemble spontaneously and then be recruited to sites of snRNA and histone mRNA synthesis [26,56]. Recent experiments using green fluorescent protein (GFP)-tagged coilin and Sm proteins are more compatible with a spontaneous assembly model, and point to the nucleolus as a potential nucleation site for coiled bodies [57,58]. Most tellingly, coilin and Sm snRNPs accumulate within nucleoli in the presence of a phosphatase-insensitive coilin mutant or the serine/threonine phosphatase inhibitor okadaic acid. Given the well-known association of coiled bodies with the nucleolar periphery (many images appear to show coiled bodies either budding off or docking onto nucleoli) and the recently described physical interaction of the nucleolar shuttling protein Nopp140 with p80 coilin [59], it seems more likely that coiled bodies form in the nucleolus.

Regardless of whether U2 snRNA genes nucleate coiled bodies *in situ* or recruit pre-formed coiled bodies, we must ask whether the interaction of nascent U2 snRNA with coiled bodies is fortuitous or functional. Although the association of specific genes with coiled bodies might simply reflect the fortuitous affinity of certain nascent RNAs for coiled body components, three facts argue that the associations are functional: the diversity of genes that exhibit such associations (U1, U2, U3 and histone genes); the functional similarities between the products of these genes (all encode small RNAs involved in RNA processing or intronless mRNA precursors that are processed by small RNAs); and the phylogenetic conservation of these associations from amphibians to mammals ([6]; M.R.F. and A.G.M, unpublished observations).

Do coiled bodies regulate U snRNA transcription?

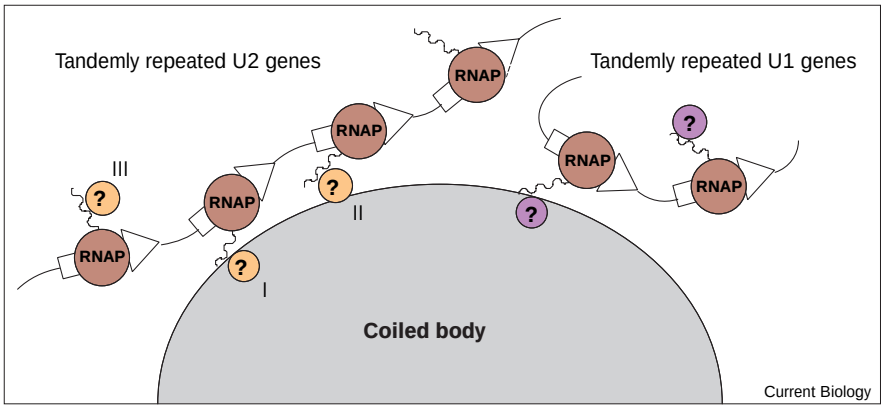
Newly transcribed U snRNAs are exported to the cytoplasm where they acquire trimethylguanosine (TMG) caps and Sm core proteins before being imported back into the nucleus [23]. The presence of TMG and Sm epitopes within coiled bodies suggests that these snRNAs have completed the cytoplasmic phase of the snRNP life cycle [2–4]. Although it is not clear whether maturing snRNPs and nucleolar snoRNPs must pass through the coiled body, at least a fraction of the nuclear snRNP and snoRNP populations associate with coiled bodies at some point in the RNP life cycle [34,36,37,60]. What then could account for association of U2 snRNA genes with a nuclear organelle containing mature (or partially mature) U2 snRNPs, and why would this association be mediated by nascent U2 snRNA, rather than by DNA or DNA-bound proteins? We suggest the unifying hypothesis [6] that coiled bodies participate in regulating U snRNA levels by an autogenous feedback loop in which a fraction of the mature (or maturing) U snRNPs return to the genes and regulate U snRNA expression (Figure 6).

Nascent snRNA transcripts could interact directly or indirectly with mature (or maturing) snRNPs within the coiled body, with snRNP-specific proteins, or with modification 'guide' RNPs that can base-pair with specific snRNA substrates [61–63] and whose protein constituents are localized in coiled bodies [8,10]. Alternatively, the maturing snRNPs may be tethered to coiled bodies via modification guide RNPs and the nascent snRNA transcripts could interact with other coiled body components. Irrespective of the precise mechanism, we speculate that an interaction between the nascent snRNA transcript and any of these components could then downregulate U snRNA synthesis by transcriptional attenuation (Figure 6). This model is not entirely unprecedented. Although transcription is more commonly regulated at the level of initiation, elongation can also be regulated negatively or positively at the DNA [64,65] or RNA level [66].

To explain why active snRNA genes are not invariably associated with coiled bodies but rather associated in direct proportion to transcriptional activity (Figure 3), we suggest that active snRNA genes are gently tethered to coiled bodies through weak interactions involving multiple transcripts (Figure 6). Higher levels of transcription would strengthen the interaction, raise the frequency of association, and increase the degree of transcriptional repression in an autoregulatory circuit. Interestingly, we find that solitary snRNA genes (for example, U11 and U12) associate with coiled bodies less frequently than do the larger U1 and U2 clusters (E. Jacobs, M.R.F. and A.G.M., unpublished observations) as expected if the association depends on multiple weak interactions.

A similar interaction between nascent RNA and coiled body components could also explain the coiled body association of: firstly, U1 genes (Figure 6; [6,33]) if nascent U1 transcripts interact with U1-specific snRNP components

Figure 6



Models for autogenous regulation of U snRNA transcription by coiled bodies. Nascent U2 snRNA associates with the coiled body surface either directly (I) or indirectly (II) through a U2 snRNP-specific protein, a U2 snRNP-associated protein (RNAP) or other coiled body component (question marks). Nascent U1 snRNA would associate with coiled bodies by analogous mechanisms. As shown in the model, we speculate that association of the nascent snRNAs with the coiled body surface would attenuate U snRNA transcription. Transcription might also be positively regulated (III) if excess factors not bound by coiled bodies could interact with the nascent snRNAs and pre-empt attenuation. If snRNPs and other coiled body components constantly associate with and dissociate from the coiled body, the coiled body surface could thus serve as a monitor of the cellular concentration of newly made snRNPs, providing the genes with a 'snapshot' of multiple macromolecular assembly processes.

within the coiled body; secondly, snoRNA genes [34] if nascent transcripts interact with coiled body components such as fibrillarin [8,67] or NAP57 [10,68]; and thirdly, histone genes if nascent transcripts interact with coiled-body-bound U7 snRNPs [6,31] which process the 3′ end of histone mRNAs [69]. Indeed, our model would explain how a single coiled body can associate simultaneously with multiple chromosomal loci (Figures 5,6) yet regulate individual U snRNP levels independently [70].

As mentioned in the Background section, coiled bodies are surprisingly heterogeneous. The explanation for this might lie in the remarkable diversity of coiled body components, including factors that participate in RNA polymerase II transcription (TFIIH, TFIIF, TBP, PTF and topoisomerase I), mRNA processing (major and minor class U snRNPs), rRNA processing (U snoRNPs of both the fibrillarin and H/ACA classes), and perhaps S-phase functions such as 3′ processing of replicative histone mRNAs by U7 snRNP. The number and morphology of coiled bodies might then reflect the unique balance of macromolecular components found in any particular cell type or line. For example, snRNPs normally partition between several nuclear compartments (nucleoli, perichromatin fibrils, interchromatin granules and coiled bodies). Cells lacking coiled bodies might be 'running on empty', unable to accumulate a sufficient excess of snRNPs or other components to form any coiled bodies at all. In a cell lacking sufficient quantities of U2 snRNP, coiled bodies might form but be deficient in U2 snRNP. In the model we propose (Figure 6), such U2-deficient coiled bodies would be unable to interact efficiently with nascent U2 snRNA, thus derepressing U2 transcription and restoring normal levels of U2 snRNP in an autoregulatory fashion. Conversely, an excess of snRNP-specific proteins could (in the absence of the mature snRNP) upregulate U snRNA transcription by binding directly to nascent RNA and blocking its interaction with the coiled body (Figure 6). A similar scenario could explain why gems appear to be distinct from coiled bodies in one particular cell line [35] but indistinguishable in many others [38]. Cells in which coiled bodies and gems are distinct would be deficient in whatever coiled body component (a specialized receptor?) enables coiled bodies and gems to intermingle. Curiously, although coiled bodies are commonly defined by the presence of p80 coilin, the protein may not be essential for coiled body formation [18] and thus cannot function as a universal glue to bind coiled body components together. Instead, we suggest that the diverse macromolecular components of coiled bodies are held together by a diverse set of weak interactions, thus enabling the coiled body to simultaneously regulate (and perhaps integrate) multiple aspects of the transcription, RNA processing and cell cycle machinery. Clearly we have much to learn about this major, but belatedly appreciated nuclear organelle.

Materials and methods

Construction of artificial tandem arrays of U snRNA genes

Tandem arrays of U1 and U2 genes were generated as described [42,43]. For cell lines bearing the 'replacement' arrays (Figure 1), the parental construct was plasmid βGLpr/ml6 [50] in which the rabbit β-globin promoter drives expression of a heterologous *Pvu*II–*Hind*III fragment derived from the first intron of the adenovirus 2 (Ad2) major late transcription unit. Replacement sequences are followed by a human U1 3′ end-formation signal extending from the last four nucleotides of the U1 coding region to the downstream *Bam*HI site. To replace the globin promoter with the U2 promoter, the replacement fragment and the 3′ end-formation signal were excised from βGLpr/ml6 as an *Nhe*I–*Bam*H I fragment, and joined in a three-way ligation to the U2 promoter (excised from plasmid mU2 as a *Bgl*II–*Sfa*NI fragment) and vector (excised from pUC18Bgl as a *Bam*HI–*Bgl*II fragment [42]. The junctions of the U2 promoter with Ad2, and Ad2 with the 3′ end-formation signal, were confirmed by sequencing.

Copy number of transfectants and RNA ratio calculations

As described previously [42], gene counting was performed by genomic blotting followed by phosphorimager analysis, and the relative transcriptional activities of the transfected genes (the RNA ratio; Figure 3) were measured by primer extension analysis. Steady-state ratios of U2:U1 were determined using equal amounts of U1 [43] and U2 [42] primers. Total RNA and labeled primers were denatured for 3 min at 90°C, annealed by slow cooling to room temperature, extended, and the products resolved by denaturing PAGE followed by phosphorimager analysis [42,43]. To avoid primer-driven reactions, primer concentrations were systematically reduced until the apparent U2 : U1 ratio was unaffected by a further twofold reduction; the resulting values were consistent with those determined directly by mass [71] or *in vivo* labeling [72]. The U2:U1 ratios obtained in this way were: mU2ΔDSEΔPSE-4, 0.99; mU2ΔDSEΔPSE-14, 0.74; mU2+CT-C03, 0.69; mU2ΔDSEΔPSE-33, 0.92; mU2ΔDSEΔPSE-43, 1.04; mU2ΔDSEΔPSE-48, 1.03; iU2-A41, 1.38; HT1080, 0.67; Raji, 0.57; BJAB, 0.92; Jurkat, 1.26; HeLa, 1.04; HEK-293, 1.20.

In situ *hybridization and immunofluorescence microscopy*

Cells were plated on chambered slides and grown overnight to 50–70% confluency. Slides were fixed and prepared for DNA FISH and immunocytochemistry as described [6]. For RNA FISH, all solutions were treated with DEPC. Prior to fixation, the cells were then rinsed in 1 × PBS at room temperature, and subsequently in cold (4°C) 1 × CSK buffer [73]. Permeabilization was carried out on ice with 0.5% Triton-X-100, 1× CSK, 2 mM vanadyl ribonucleoside complex (VRC) for 1 min. Following a quick rinse in 1 × PBS, the slides were fixed and hybridized as above, with the following modifications. Slides were stored in 70% ethanol until use. For RNA detection, cells were dehydrated in an ethanol series and hybridized without denaturation [73]. The probe solution (total volume 15 μl) for each slide consisted of 80 ng replacement construct nick-translated with biotin-16-dUTP, 2 μg C_0t1 DNA (Gibco/BRL), 9 μg sonicated, denatured salmon DNA and 20 mM VRC. Microscopy and imaging were as described [6].

Cell scoring and coiled body association frequencies

A dual bandpass (FITC and Texas red) filter set (Chroma Tech.) was used for scoring interphase nuclei. The numbers of coiled bodies and DNA loci were scored visually, and the number of signals that were at least partially overlapping was counted. A Cy5 (infrared bandpass) filter set (Chroma Tech.) was used to image the endogenous U1 and U2 gene probes. The number of endogenous versus exogenous loci that associate with coiled bodies was determined by first locating a coiled body–gene pair in the dual-pass filter and then imaging the individual red and green signals. Then the filter was switched to the Cy5 channel in order to determine whether the gene signal was endogenous (signal present) or exogenous (no signal). Cell lines containing large arrays of the iU2 repeat could be scored visually, as the signals derived from the artificial arrays could be distinguished by morphology (artificial array signals were much bigger). Note that the frequency of

association of U1, U2 and histone genes with coiled bodies is also dependent on cell type. For example, HeLa strains adapted to adherent growth (HeLa-ATCC) or suspension culture (HeLa-JS1000) exhibit different frequencies of coiled body–*RNU2* association (for example, ~70% of HeLa-ATCC and 20% of HeLa-JS1000 cells had at least one overlapping coiled body–*RNU2* signal pair). These two different HeLa strains also displayed markedly different numbers of coiled bodies (5.1 versus 1.9) and U2 loci (2.8 versus 1.5); see Frey and Matera [6] for details. Furthermore, the frequency of association is not due simply to growth conditions; the relative levels of coiled body–*RNU2* association are maintained when the adherent strain is adapted to spinner culture or the suspension culture to adherent growth (M.R.F. and A.G.M., unpublished observations).

In situ *discrimination of endogenous and exogenous snRNA genes*

PCR primers flanking the artificial minigene constructs for *RNU2* (see Figure 1) and *RNU1* were used to amplify intergenic sequences present in genomic DNA but absent from the artificial arrays. The *RNU2* primers (RNU2-1: 5′-CCTCTGCCGCTCTTTTGGGTCTCA-3′; RNU2-2: 5′-CCTGCGTCTCCTGCGGCTTCTGTG-3′) and *RNU1* primers (RNU1-1: 5′-AGATTGGCGGTTGAGTGGCAGAA-3′; RNU1-2: 5′-CAGGGCATGTAACAGTGGGTGAATA-3′) produced 3.7 kb and 4.1 kb products, respectively, using various long-range PCR kits (Stratagene, Boehringer Mannheim) and 32 cycles per reaction. These PCR products were then labeled by nick translation with Cy5–dUTP (Amersham) and used for specific hybridization to the wild-type *RNU2* or *RNU1* loci. To demonstrate that artificial U2 genes associate with bona fide coiled bodies, cell lines A37 and A41, containing iU2 arrays (Figure 3), were hybridized with biotinylated antisense 2′-OMe U2 or U7 oligonucleotides and detected with fluorescently labeled avidin. For detection of other coiled body components, antibodies against Nopp-140 (monoclonal antibody RE10, gift of T. Meier), fibrillarin (monoclonal 72B9, gift of E. Chan) or U2B″ (monoclonal 4G3, gift of H. Salz) were used instead of the antisense oligonucleotides.

Hybridization probes

The U1 (1p36), U2 (17q21) and U3 (17p11) probes were described previously [6,34]. BAC and P1 clones corresponding to the histone gene clusters on chromosomes 1q21 and 6p21 and the U7 (12p13) and U12 (22q13) snRNA genes were isolated by PCR screening (W. Wu and A.G.M., unpublished) of a human BAC library (Research Genetics). Each probe was labeled with biotin-16–dUTP (80 ng per probe per slide) and a mixture of all seven probes was ethanol-precipitated along with 3 μg C_0t1 and 9 μg salmon sperm DNA before use. Hybridization, detection, and simultaneous coilin immunofluorescence were carried out as described [6].

Supplementary material

A figure showing the effects of RNA polymerase II disruption on the colocalization of U2 genes with coilin foci is published with this paper on the internet.

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Supplementary material

Association of snRNA genes with coiled bodies is mediated by nascent snRNA transcripts

Mark R. Frey, Arnold D. Bailey, Alan M. Weiner and A. Gregory Matera

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Figure S1

Global inhibition of RNA polymerase II disrupts the coiled body–*RNU2* association. HeLa cells were treated with 5 μg/ml α-amanitin for 5 h and then processed for immunofluorescence and FISH. (a–c) Controls demonstrating the characteristic enlargement of interchromatin granule clusters as shown by anti-U2 B″ antibodies – green in (b). Images of the same cell nucleus are shown in (a) with DAPI (blue) and in (c) with anti-p80 coilin (red). (d,e) Treated cells were hybridized with (e) the *RNU2* probe (green) and anti-p80 coilin antibody (red). The same nuclei stained with DAPI are shown for reference in (d). Note that in (e) much of the coilin staining forms nucleolar cap-like structures and that the merged green and red fluorescence images do not overlap.