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Coiled Bodies and U2 snRNA Genes Adjacent to Coiled Bodies Are Enriched in Factors Required for snRNA Transcription

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A significant percentage of the gene clusters that contain the human genes for U1 small nuclear RNA (snRNA) or for U2 snRNA have been found associated with small nuclear domains, known as coiled bodies. We show here, by immunofluorescent labeling of human cells, that coiled bodies are enriched in factors required for the transcription of these snRNA genes. The 45-kDa γ-subunit of the transcription factor, proximal element sequence-binding transcription factor (PTF), which is specific for the snRNA genes, was found in high concentrations in coiled bodies, along with the general transcription factor TATA-box binding protein and a subset of RNA polymerase II. We show that the transcription factors and RNA polymerase II are concentrated in irregularly shaped domains that not only overlap with coiled bodies but also extend to their immediate surroundings. Fluorescent in situ hybridization showed that these domains can overlap with U2 snRNA genes adjacent to coiled bodies. In addition, we found the domains to contain newly synthesized RNA, visualized by 5-bromo-uridine triphosphate labeling. Our data suggest that coiled bodies are involved in the expression of snRNA genes, which leads us to propose the model that coiled bodies are associated with snRNA genes to facilitate and regulate their transcription. These findings point to a general principle of higher order organization of gene expression in the nucleus.

INTRODUCTION

Recently, the genes coding for U1 and U2 small nuclear RNA (snRNA) have been found associated with a specific nuclear domain, known as the coiled body (Frey and Matera, 1995; Smith *et al.*, 1995). Coiled bodies are small spherical structures, one to five per nucleus, with a diameter of 0.2–1.0 μm. They are evolutionarily conserved from plants to mammals, indicating that they have a crucial role in the nucleus (for a review see Gall *et al.*. 1995). Over the years, many nuclear factors have been found concentrated in coiled bodies. Among these are nucleolar constituents, such as fibrillarin (Raška *et al.*, 1990), Nopp140 (Meier and Blobel, 1992), and U3 small nucleolar RNA (snoRNA) (Jiménez-García *et al.*, 1994), and nucleoplasmic factors, such as TFIIH, TFIIF (Grande *et al.*, 1997; Jordan *et al.*, 1997), CstF, and CPSF (Schul *et al.*. 1996), and small nuclear ribonucleoprotein particles (snRNPs) U1, U2, U4/U6, and U7 (Carmo-Fonseca *et al.*, 1992; Wu and Gall, 1993; Frey and Matera, 1995). The protein p80-coilin is especially enriched inside coiled bodies (Andrade *et al.*, 1991; Raška *et al.*, 1991) and is a hallmark for this nuclear domain. Coiled bodies can be disrupted by inhibiting transcription or by heat shock, indicating that they are dynamic structures that are influenced by the nuclear activity of the cell (Carmo-Fonseca *et al.*, 1992). Despite the large amount of data available on coiled bodies, their function is still unclear (for a review see Lamond and Carmo-Fonseca, 1993).

The recent detection of U1 and U2 snRNA genes adjacent to coiled bodies has opened new avenues of investigation on the possible function of these enigmatic nuclear structures. The snRNA genes are present as clustered multiple copies in the genome. In

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humans there are about 30 copies of the U1 gene (Lund and Dahlberg, 1984) and 10–20 copies of the U2 gene (Van Arsdell and Weiner, 1984) per haploid genome, clustered on chromosome 1 and chromosome 17, respectively. The snRNA genes are highly transcribed, placing the snRNA promotors among the most active in the cell (Dahlberg and Lund, 1988). It is unclear, however, what influence coiled bodies have on the snRNA genes that they are associated with, and whether they play any role in the transcription or maturation of snRNA.

Transcription of the mammalian snRNA genes is directed by two promotor elements: the proximal sequence element (PSE)[1] and the distal sequence element (DSE). As the name suggests, the PSE lies closest to the gene, around position −60 to −50 of the start site and is essential for transcription initiation. The DSE, at about −220 from the start site, functions as an enhancer. The U1 through U5 snRNA genes are transcribed by RNA polymerase II, but lack a TATA box; basal transcription of these genes is directed by the PSE (for a review see Lobo and Hernandez, 1994). The PSE of the human snRNA genes is specifically recognized by a PSE-binding transcription factor (PTF) (Murphy *et al.*, 1992), also known as the PBP (Waldschmidt *et al.*, 1991) or the SNAPc complex (Sadowski *et al.*, 1993). PTF has been isolated as a stable complex of four subunits: PTFα (180 kDa), PTFβ (55 kDa), PTFγ (45 kDa), and PTFδ (44 kDa) of which the last three have been cloned and sequenced. Additionally, the general transcription factor TATA-box binding protein (TBP) is found in this complex in substoichiometric amounts. It has been shown that TBP interacts with the PTFγ and PTFδ subunits and is necessary for snRNA gene expression (Yoon *et al.*, 1995; Yoon and Roeder, 1996; Henry *et al.*, 1995, 1996; Bai *et al.*, 1996; Sadowski *et al.*, 1996).

The percentage of the U1 and U2 snRNA gene clusters that is associated with a coiled body is dependent on the cell type and ranges from 20% to 90% in different cell lines. However, there are always snRNA gene loci not associated with a coiled body (Frey and Matera, 1995; Smith *et al.*, 1995). Why some U1 or U2 snRNA genes are associated with a coiled body and others are not is unknown. To gain insight into the functional relationship between the snRNA genes and the coiled body, we investigated whether the factors required for expression of the snRNA genes are present at the coiled body. Using immunofluorescent double labeling in combination with confocal laser scanning microscopy, we found that PTFγ, TBP, and hypophosphorylated RNA polymerase II are concentrated in and around coiled bodies, overlapping with the U2 snRNA gene clusters. These findings show that the snRNA genes coincide with their transcription factors at the coiled body. We propose that the high concentrations of transcription factors allow the snRNA genes to be efficiently transcribed at the periphery of the coiled body.

[1] Abbreviations used: BrUTP, 5-bromo-uridine triphosphate; DSE, distal sequence element; PSE, proximal sequence element; PTF, PSE-binding transcription factor.

MATERIALS AND METHODS

Cell Culture

T24 cells (from human bladder carcinoma), HeLa cells (from human cervical carcinoma), and CaCo cells (from human colon carcinoma) were grown on circular glass coverslips at 37°C under a 10% CO_2 atmosphere in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 1% glutamine (Life Technologies), 10% fetal calf serum (Life Technologies) and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin [Life Technologies]). Cells were used at 50–70% confluency.

Immunofluorescence Labeling

All steps were performed at room temperature unless stated otherwise. Coverslips with attached cells were rinsed once in PBS and incubated with 2% paraformaldehyde in PBS for 15 min. After fixation, cells were rinsed twice with PBS and permeabilized with 0.5% Triton X-100 (Sigma Chemical, St. Louis, MO) in PBS for 5 min. Cells were subsequently rinsed twice in PBS, incubated in PBS containing 100 mM glycine (Sigma) for 10 min, and incubated for 10 min in PBG (PBS containing 0.5% BSA [Sigma] and 0.05% gelatin from cold-water fish skin [Sigma]).

For immunolabeling the following antibodies were used: polyclonal antibody from rabbit against PTFγ (gift from Dr. R.G. Roeder)(Yoon and Roeder, 1996), monoclonal antibody 1C2 from mouse against TBP (gift from Drs. L. Tora and P. Chambon) (Lescure *et al.*, 1994), monoclonal antibody from mouse against the p62 subunit of TFIIH (gift from Dr. J.M. Egly) (Schaeffer *et al.*, 1994), polyclonal antibody from rabbit against the RAP74 subunit of TFIIF (gift from Dr. S. Kitajima) (Yonaha *et al.*, 1993), monoclonal antibody H5 from mouse (gift from Dr. S.L. Warren) (Bregman *et al.*, 1995), and monoclonal antibody 8WG16 from mouse (gift from Dr. N.E. Thompson) (Thompson *et al.*, 1989) against the RNA polymerase large subunit, polyclonal antibody 204/5 from rabbit (gift from Dr. A.I. Lamond) (Bohmann *et al.*, 1995a), and monoclonal antibody 5P10 from mouse (gift from Dr. M. Carmo-Fonseca) against p80-coilin, monoclonal antibody against CstF 64 kDa, from mouse (gift from Drs. Y. Takagaki and J.L. Manley) (Takagaki *et al.*, 1990), polyclonal antibody RF12 from rabbit against Nopp140 (gift from Dr. U.T. Meier) (Meier and Blobel, 1992), monoclonal antibody 72B9 from mouse against fibrillarin (gift from Drs. K.M. Pollard and E.M. Tan) (Takeuchi *et al.*, 1995), and anti-BrdU polyclonal antibody from rat (Seralab, Crawley Down, United Kingdom).

Fixed cells were incubated overnight at 4°C or for 2 h at room temperature with primary antibodies diluted in PBG. Subsequently, cells were washed four times for 5 min in PBG and incubated with secondary antibodies diluted in PBG for 1.5 h. Secondary antibodies (from Jackson ImmunoResearch Labs, West Grove, PA) used were: donkey anti-mouse IgG coupled to 4,6-dichlorotriazinyl aminofluorescein (DTAF) or Cy3; donkey anti-rabbit IgG coupled to FITC or Cy3; and donkey anti-rat IgG coupled to Cy3.

After labeling, cells were washed two times for 5 min in PBG and two times for 5 min in PBS followed by incubation in PBS containing 0.4 μg/ml Hoechst 33258 (Sigma) for 5 min. All coverslips were mounted in Vectashield (Vector Laboratories, Burlingame, CA).

Microinjection

T24 cells were microinjected with 5-bromo-uridine triphosphate (BrUTP) as described by Wansink *et al.* (1993, 1994). After microin-

jection, cells were cultured for 5 min at 37°C and subsequently fixed and labeled as described above.

Fluorescent in Situ Hybridization in Combination with Immunofluorescence Labeling

When immunofluorescence labeling was combined with in situ hybridization, the following adaptations and additions to the above protocol were implemented. PBG was substituted with PBH (PBS containing 0.1 mg/ml nuclease-free acetylated BSA [Sigma] and 0.1 μg/ml herring sperm DNA). After primary and secondary antibody labeling, the cells were fixed for 5 min with 2% formaldehyde in PBS, washed twice in PBS, incubated 10 min in 100 mM glycine in PBS, and washed in PBS.

Cells were dehydrated by subsequent incubations in 70%, 90%, and 100% ice-cold ethanol for 4 min per incubation and air dried. Genomic DNA was denatured by incubating the coverslips in 2×SSC containing 70% formamide (pH 7.2) at 80°C for 5 min. Immediately after, the cells were treated with the 70%, 90%, and 100% ice-cold ethanol for 4 min each and air dried. The cells were incubated overnight in probe solution at 37°C.

The probe was produced from a human genomic clone containing ~6 kb of the RNU2 locus at 17q21 (gift from Drs. A.G. Matera and A.M. Weiner) by nick translation using digoxigenin-labeled dUTP essentially as described by Rigby *et al.* (1977) and Langer *et al.* (1981). The probe was heat denatured in 70% deionized formamide together with COT-1 DNA (Boehringer) at 80°C for 10 min. The final probe solution contained 2×SSC, 50% formamide, 10% dextran sulfate, COT-1 and herring sperm DNA, and the labeled probe.

After incubation with probe solution, the coverslips were washed three times for 5 min in 2×SSC containing 50% formamide (pH 7.2) at 39°C and three times for 5 min in 1×SSC at room temperature. The cells were washed twice in PBS and incubated for 30 min in PBH. Subsequently, the coverslips were incubated for 60 min in PBH containing FITC-conjugated anti-digoxigenin antibody (Sigma). The cells were then washed four times in PBS. The cells were stained with Hoechst and embedded and mounted as described above.

Confocal Laser Scanning Microscopy and Image Analysis

Images of double labeled cells were produced on a Leica confocal laser scanning microscope with a 100×/1.35 oil immersion lens. A dual wavelength laser was used to excite green (DTAF or FITC) and red (Cy3) fluorochromes simultaneously at 488 nm and 514 nm, respectively. The fluorescence signals from the two fluorochromes were recorded simultaneously. Optical cross-talk was quantified and subtracted as described previously (Manders *et al.*, 1992). Image analysis was performed using Scil-Image software, developed at the University of Amsterdam (Van Balen *et al.*, 1994). Images were subjected to a restoration procedure to correct for diffraction-induced distortions using a measured point spread function (Van der Voort and Straster, 1995).

Western Blotting

Total T24 protein extract was prepared by harvesting T24 cells in ice-cold sample buffer (2% SDS, 0.1 M Tris, pH 6.8, 4% β-mercaptoethanol, 15% glycerol) containing 1 mM PMSF, 0.1 mM EGTA, 1.2 mM benzamidine, and 1 μg/ml leupeptin. The protein extract was heat denatured, subjected to SDS-PAGE on a 12% gel (Laemmli, 1970), and transferred to nitrocellulose (Towbin *et al.*, 1979). The nitrocellulose blot was incubated for 2 h with 1% blocking reagent (Boehringer), washed in PBGTNa (PBS containing 0.5% BSA [Sigma], 0.05% gelatin from cold-water fish skin [Sigma], 0.05% Tween 20, and 300 mM NaCl), incubated overnight with primary antibody diluted in PBGTNa, washed four times for 5 min in PBGTNa, incubated 2 h with the secondary antibody goat anti-mouse coupled to alkaline phosphatase (Jackson ImmunoResearch) or goat anti-rabbit antibody coupled to alkaline phoshatase (Bio-Rad, Richmond, CA), washed two times for 5 min in PBGTNa and two times for 5 min in PBS. Blots were stained by incubation in 100 μg/ml nitrotetrazolium blue chloride (NBT), 50 μg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in buffer (0.1 M Tris, 0.1 M NaCl, 5 mM $MgCl_2$ [pH 9.2]).

RESULTS

PTFγ and TBP Are Concentrated in and around the Coiled Body

To study the spatial distribution of the two transcription factors necessary for activation of the snRNA promotor, TBP and PTF, we performed immunofluorescent labeling experiments in combination with confocal laser scanning microscopy. All antibodies we used were shown to be monospecific based on Western blot analysis (Figure 1). A monoclonal antibody against TBP and a polyclonal antibody against the PTFγ subunit were used on human T24, HeLa, and CaCo cells. All findings were similar for the three cell types. TBP showed an overall granular nuclear distribution, in agreement with its general role in the transcription of all genes. TBP was additionally found concentrated in one to three foci in the nucleoplasm (Figure 2A). Labeling of PTFγ showed that this transcription factor was also distributed throughout the nucleus in low concentrations in addition to a few brightly labeled nuclear foci (Figure 2B). Furthermore, in a small percentage of the cells, there was also a larger PTFγ-labeled domain at the periphery of the nucleolus (our unpublished observations), comparable to the perinuclear domains enriched in heteroge-

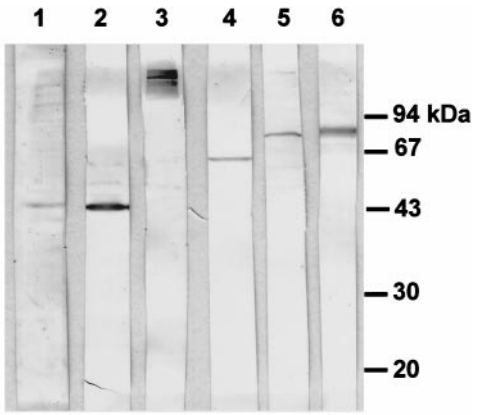

Figure 1. Western blot confirming the monospecificity of the antibodies used. Only bands at the expected position are observed with antibodies against the following proteins: PTFγ, 45 kDa (lane 1); TBP, 44 kDa (lane 2); RNA polymerase II large subunit, 240 kDa (lane 3); TFIIH p62, 62 kDa (lane 4); TFIIF RAP74, 74 kDa (lane 5); and p80-coilin, 80 kDa (lane 6). The antibody 8WG16 against the RNA polymerase II large subunit (lane 3) labels multiple bands due to phosphorylation of the protein.

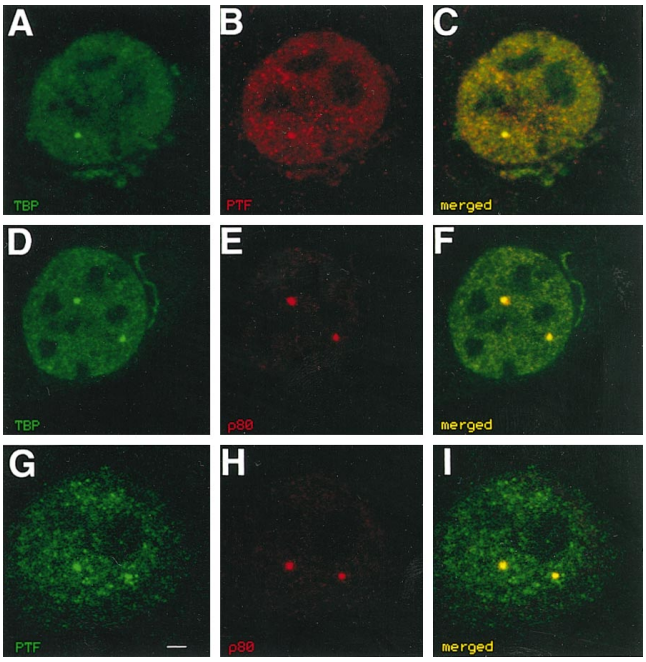

Figure 2. Optical sections of immunofluorescently double-labeled T24 cells are shown. (A and D) Labeling of TBP shows the transcription factor to be distributed throughout the nucleus and concentrated in a few small nuclear domains. (B and G) Labeling for PTFγ gives a similar overall distribution and is concentrated in a few nuclear foci. (C) When the labeling patterns are compared, the brightly labeled domains colocalize. (E and H) Labeling for p80-coilin reveals coiled bodies. When the p80-coilin labeling is compared with the TBP labeling (F) or the PTFγ labeling (I), the transcription factor-enriched domains colocalize with coiled bodies. Bar, 2 μm.

neous nuclear ribonucleoprotein I (hnRNP I) hnRNP L, and Oct1 (Piñol-Roma *et al.*, 1989; Ghetti *et al.*, 1992; Grande *et al.*, 1997). The perinucleolar PTF domain has recently been characterized by Pombo *et al.* (1998) but was shown not to be associated with the snRNA genes.

Double labeling experiments showed that the foci labeled by TBP and PTFγ overlapped (Figure 2C), and both factors were thus found concentrated together in these small nuclear domains. Since the size, shape, and number of the foci were reminiscent of a nuclear structure known as the coiled body, we performed double-labeling experiments with antibodies against the protein p80-coilin, a hallmark for coiled bodies (Raška *et al.*, 1991). This revealed that the foci labeled by the two transcription factors colocalized with coiled bodies (Figure 2, D–I). We used image restoration of three-dimensional confocal recordings to obtain images of sufficiently high effective resolution to further study this spatial correlation in detail. It became clear from these images that while the coiled bodies, visualized by the p80-coilin labeling, were spherical and compact, the domains enriched in TBP and PTFγ were more extended, forming larger and more irregularly shaped domains. These transcription factor-enriched domains overlapped with the coiled body but also extended beyond the coiled body and could often be seen adjacent to it or surrounding it (Figure 3, A–D). Apparently, there is a complex spatial relationship between coiled bodies and the domains enriched in TBP and PTFγ.

Other Basal Transcription Factors and a Subset of RNA Polymerase II Molecules Are Also Concentrated in and around the Coiled Body

It has been reported that components of the general transcription factors TFIIH and TFIIF are concentrated in foci that overlap with coiled bodies (Grande *et al.*, 1997; Jordan *et al.*, 1997). We have reproduced these observations by using antibodies against the p62 subunit of TFIIH and against the RAP74 subunit of TFIIF, in combination with the p80-coilin labeling. Closer inspection revealed that, also, the foci enriched in TFIIH p62 and TFIIF RAP74 are not as compact as the coiled body and extend beyond its boundaries (Figure 3, E and F). To test whether this distribution was a general phenomenon for components of the coiled body, we compared the p80-coilin labeling with the labeling of coiled-body proteins fibrillarin and Nopp140. Apart from the nucleolar labeling, both proteins were solely concentrated in compact domains of roughly the same shape and size as the p80-coilin–labeled coiled body with which they colocalized (Figure 3, G and H).

The antibody H5 recognizes the hyperphosphorylated form of the carboxy-terminal domain (CTD) of the large subunit of RNA polymerase II. Immunofluorescent labeling of cells using this antibody gives a dotted nuclear distribution (Zeng *et al.*, 1997; Grande *et al.*, 1997). These dots were never found overlapping with a coiled body, although a few of them could often be seen in close contact with the coiled body (Figure 4A). The antibody 8WG16 detects various phosphorylated forms, but preferentially binds to the hypophosphorylated forms of RNA polymerase II (Bregman *et al.*, 1995). Interestingly, this antibody has already been reported to label two to five bright dots per nucleus, in addition to a finely punctated distribution throughout the nucleoplasm (Bregman *et al.*, 1995). In a double labeling with p80-coilin, the 8WG16 domains were often tightly associated with the coiled body. The 8WG16 domains were irregularly shaped and usually only partially overlapped with the coiled body (Figure 4B). Sometimes the 8WG16 domain seemed to embrace the coiled body, having little overlap with it. Double labeling of 8WG16 with PTFγ and TBP showed that the domains enriched in these proteins strongly overlapped.

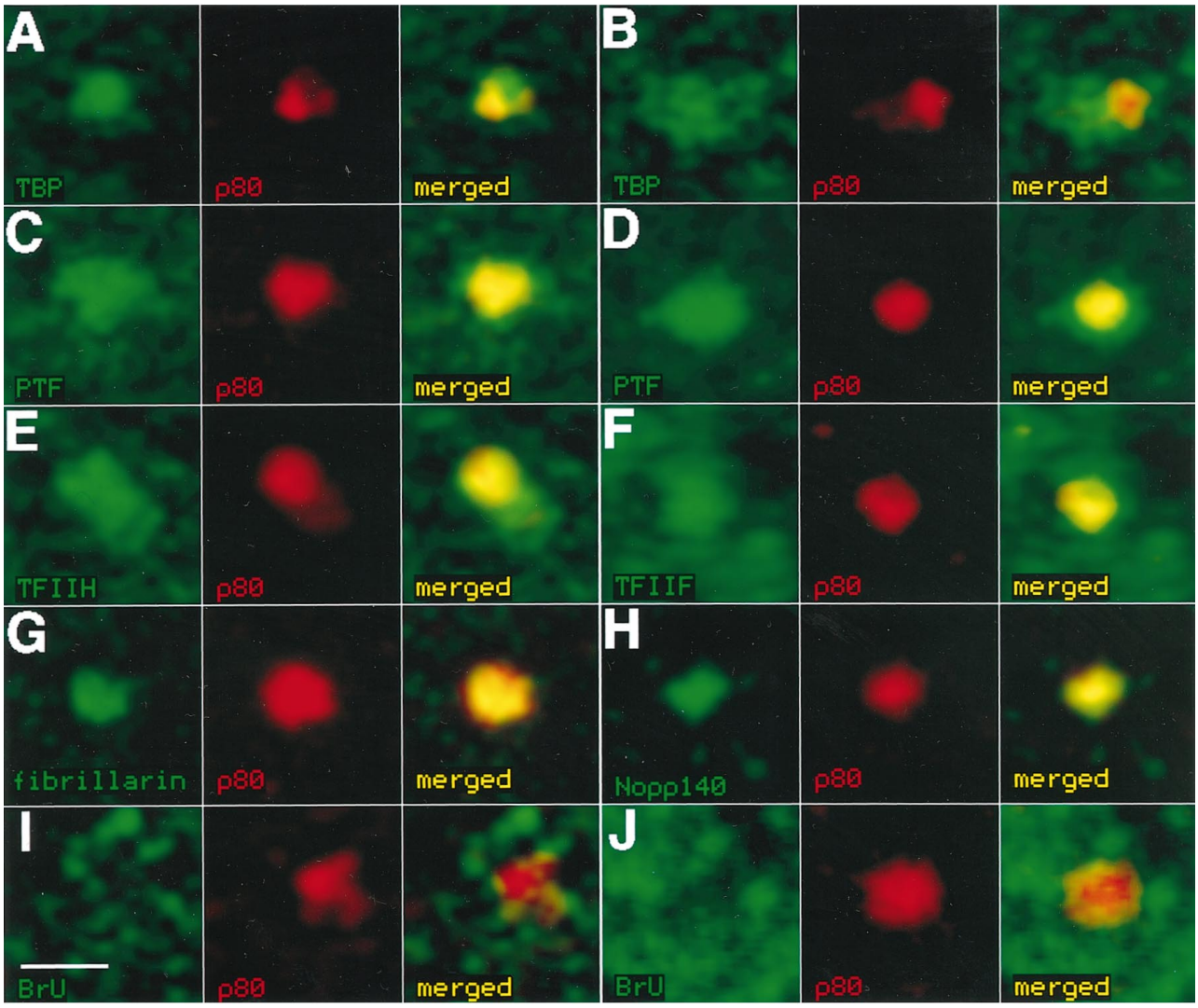

Figure 3. Magnified areas of optical sections of immunofluorescently double-labeled nuclei are shown. Coiled bodies, visualized in red by p80-coilin labeling, are compact spherical domains. Labeling in green of TBP (A and B), PTFγ (C and D), TFIIH p62 (E), and TFIIF RAP74 (F) shows that these transcription factors are concentrated in larger, more irregularly shaped domains. Merging the images reveals that the transcription factor domains partially overlap with the coiled body but also extend to the immediate surrounding of the coiled body. Labeling of other constituents of the coiled body, i.e., fibrillarin (G) and Nopp 140 (H), shows that these proteins are confined to the coiled body and do not extend beyond its boundary. Sites of RNA synthesis are visualized by incorporation of bromo-uridine (BrU) for 5 min (I) or 20 min (J) (green). Although BrU-labeled transcription sites are not observed inside coiled bodies, they can be found associated with the periphery of coiled bodies. Bar, 1 μm.

Domains associated with coiled bodies that we described previously, referred to as cleavage bodies because they are enriched in the RNA 3′- cleavage factors CstF 64 kDa and CPSF 100 kDa (Schul *et al.*, 1996), were regularly found colocalizing with the transcription factor domains. This is not unexpected because both structures are associated with coiled bodies. However, the cleavage bodies and the domains enriched in TBP, PTFγ, and RNA polymerase II were also observed only partially overlapping or juxtaposed. We did not observe a clear correlation between the labeling patterns of both domains at the periphery of coiled bodies. We therefore concluded that the domains enriched in basal and snRNA gene-specific transcription factors did not correspond to cleavage bodies, although it is very possible that both domains do have functional interactions. These data suggest that coiled bodies form centers where various elements of the basal transcription and processing machinery are concentrated.

Domains Enriched in RNA Polymerase II and Transcription Factors Overlap with the U2 snRNA Gene Loci

It has been reported that the U1 and U2 snRNA gene clusters are frequently found adjacent to coiled bodies (Frey and Matera, 1995; Smith *et al.*, 1995). Using a genomic probe for the human U2 snRNA gene (RNU2), we performed fluorescent in situ hybridization in combination with immunofluorescent labeling to confirm the association of the gene locus with coiled bodies in T24 cells. In this cell type, about 30% of the coiled bodies were found adjacent to an RNU2 gene locus (Figure 4C). Since the cells contained on average 1.5 times more RNU2 gene loci than coiled bodies, the percentage of RNU2 loci involved in pairing with a coiled body was proportionally lower, i.e., about 20%.

PTF is a specific transcription factor for the snRNA genes, and TBP and RNA polymerase II are also required for the transcription of these genes (Yoon *et al.*, 1995; Henry *et al.*, 1995). We therefore wanted to investigate the spatial relationship between the coiled body, the RNU2 gene locus, and the factors necessary for snRNA transcription. Unfortunately, the antibody against TBP no longer gave a signal when combined with the in situ hybridization protocol. The 8WG16 and PTFγ labeling continued to show the bright foci, although the labeling patterns were less defined, probably due to the deteriorated morphology of the cells caused by the in situ hybridization conditions. The 8WG16 and PTFγ foci partially or completely overlapped with 10–20% of the U2 snRNA gene clusters (Figure 4, D and E). Because the TBP foci colocalized with the 8WG16 and PTFγ foci, it is reasonable to conclude that TBP foci also overlap with the gene for U2 snRNA. Interestingly, when the U2 locus was compared with TFIIH p62, the brightly labeled dots in both signals also occasionally overlapped. Although it is not known whether TFIIH is involved in transcription of the snRNA genes, these observations suggest TFIIH might play a role in this process.

As a control, we compared the distribution of the RNU2 gene loci with the aforementioned cleavage bodies. Cleavage bodies are enriched in factors required for 3′-cleavage and polyadenylation of mRNA and are frequently found overlapping with or next to coiled bodies (Schul *et al.*, 1996). Because snRNA transcripts have a different 3′-processing than mRNAs (Dahlberg and Lund, 1988), cleavage bodies and the RNU2 loci would not be expected to overlap. Double labeling experiments confirmed that although cleavage bodies were often found close to the RNU2 loci, as predicted from their shared association with coiled bodies, they were not found overlapping in any of the 50 nuclei investigated. This indicates that the association between the RNU2 gene loci and the domain enriched in transcription factors and RNA polymerase II is specific and not coincidental.

We have shown that PTFγ, TBP, TFIIH p62, TFIIF RAP74, and a hypophosphorylated form of RNA polymerase II are concentrated together in an irregularly shaped domain in and around the coiled body. In contrast, the RNU2 gene loci were often observed adjacent to a coiled body but never found overlapping with it. This implies that it is the part of the domain just outside the coiled body that overlaps with the gene. This spatial organization makes the immediate surroundings of the coiled body a likely place for snRNA gene transcription.

The RNA Polymerase II-enriched Domains Contain Newly Synthesized RNA

We wanted to investigate whether the U2 snRNA gene is active when it is present next to a coiled body. However, since U2 snRNA is stably present in high concentrations in the nucleus, complexed in the U2 snRNP splicing factor, it has not been possible to detect the nascent transcript by in situ hybridization.

Active transcription sites can be studied by microinjecting cells with BrUTP that is incorporated into newly synthesized RNA and can be immunofluorescently detected. This labeling results in a punctated nuclear pattern, which visualizes the sites of RNA synthesis in the nucleus (Wansink *et al.* 1993; Jackson *et al.*, 1993). Various studies have reported that coiled bodies do not contain newly synthesized RNA (Moreno Diaz de la Espina *et al.*, 1982; Raška, 1995; Schul *et al.*, 1996; Jordan *et al.*, 1997). When we microinjected T24 cells with BrUTP and double labeled

Figure 4 (facing page). Optical sections of immunofluorescently double-labeled cells are shown. (A) Labeling with the H5 antibody against the hyperphosphorylated form of RNA polymerase II (green) reveals a punctated pattern throughout the nucleus. A combined p80-coilin labeling of coiled bodies (red) shows that the H5 dots do not overlap with the coiled body, although a number of dots can be found at the periphery of the coiled body (compare separate channels in magnified area). (B) Labeling with 8WG16 antibody against the hypophosphorylated form of RNA polymerase II (green) also reveals a punctated pattern in addition to a few brightly labeled domains. Double labeling with p80-coilin shows that these domains are closely associated with coiled bodies, similar to the transcription factor-enriched domains (see Figure 3) (compare separate channels in magnified area). (C) Immunofluorescent labeling for p80-coilin (green) was combined with genomic in situ hybridization, visualizing the U2 snRNA gene loci (red). A coiled body could be found adjacent to a U2 snRNA gene locus in about 30% of the cases. (D) Brightly labeled 8WG16 domains (green) could be found colocalizing with U2 snRNA gene loci (red). (E) Also domains enriched in the transcription factor PTFγ (green) were found overlapping with some U2 snRNA gene loci (red). (F) Newly synthesized RNA was visualized by microinjecting cells with BrUTP (red). Double labeling with 8WG16 (green) shows that there is active transcription inside the domain enriched in hypophosphorylated RNA polymerase II (compare separate channels in magnified area). Bar, 2 μm.

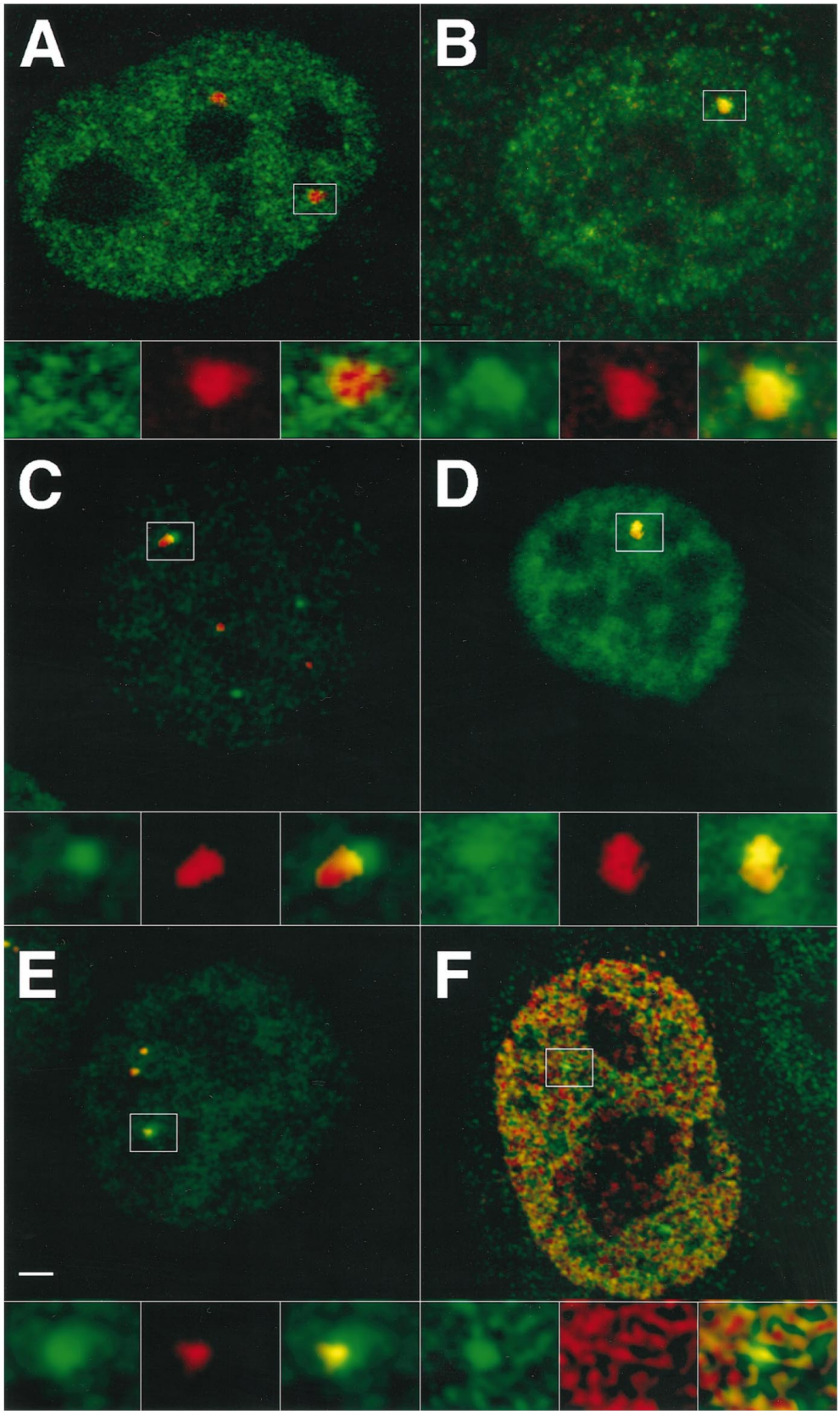

Figure 4.

them with p80-coilin, we observed that of the many BrU-labeled domains there were always a few in close contact with the coiled body (Figure 3, I and J) (also described by Raška [1995] and Jordan *et al.* [1997]). BrUTP-microinjected cells, double labeled with 8WG16, often showed one or more BrU-labeled dots inside the brightly labeled 8WG16 domain (Figure 4F). This shows that the 8WG16 domain does contain one or more active genes, although it cannot be determined whether this includes the snRNA genes.

Our data indicate that the transcription factors PTF and TBP, together with RNA polymerase II and active transcription, have an intimate relationship with the coiled body and the associated snRNA genes.

DISCUSSION

The role of coiled bodies in nuclei of various organisms has remained as much of a mystery as when Ramón Cajal (1903) described them as "round little bodies situated at a distance from the nucleolus." The discovery of Frey and Matera (1995) and Smith *et al.* (1995) that certain genes are preferentially associated with coiled bodies has opened new avenues of investigation on this elusive nuclear domain. A substantial percentage of the U1 and U2 snRNA gene clusters were found in close contact with a coiled body. It has remained unclear, however, what the influence of coiled bodies is on these genes and whether they play any role in the expression of these genes. Frey and Matera (1995) have suggested that the coiled bodies might represent sites at which snRNA transcription is being repressed. However, our data indicate that coiled bodies may function as organizing centers where snRNA genes are efficiently transcribed.

We have used immunofluorescent double labeling in combination with confocal laser scanning microscopy to investigate the spatial organization of factors involved in the transcription of snRNA genes. Three components, known to be essential for snRNA transcription, were found concentrated in coiled bodies. The snRNA gene-specific transcription factor subunit PTFγ, the TATA-box binding protein TBP, and the hypophosphorylated form of the large subunit of RNA polymerase II were found concentrated together in small irregularly shaped domains, which not only partially colocalized with the coiled body but also extended to the area around it. RNA polymerase II is responsible for the transcription of the snRNA genes U1 to U8, with the exception of the RNA polymerase III-transcribed U6 snRNA. The CTD of the large subunit of RNA polymerase II can be phosphorylated to various degrees. Hypophosphorylated RNA polymerase II has been shown to be involved in the formation of the initiation complex (Lu *et al.*, 1991; Chesnut *et al.*, 1992), and subsequent phosphorylation of the CTD is believed to release the polymerase from the initiation complex resulting in transcription of the gene (Payne *et al.*, 1989; Laybourn and Dahmus, 1990; for a review see Nikolov and Burley, 1997). Labeling experiments with the antibody 8WG16 revealed that the hypophosphorylated form is present in high concentration in and around coiled bodies. Labeling with the H5 antibody showed that the hyperphosphorylated form of RNA polymerase II, although not present inside coiled bodies, is found concentrated in dots associated with the periphery of the coiled bodies. Interestingly, the enzyme responsible for phosphorylation of the RNA polymerase II CTD, the cdk7 subunit of TFIIH, was also found concentrated in coiled bodies (Jordan *et al.*, 1997). Evidently, hypophosphorylated RNA polymerase II, together with other components involved in forming transcription initiation complexes, i.e., TBP and PTFγ, is concentrated in and around coiled bodies. This exceptional accumulation of transcription factors can be explained by the high promotor activity of the snRNA genes. On average, each U1 and U2 snRNA gene is transcribed every 2–4 s (Dahlberg and Lund, 1988). With tens of these genes per cluster, the snRNA gene clusters have a particularly high demand for transcription initiation complexes. These observations show that several elements necessary for efficient transcription of snRNA genes are concentrated together in and around the coiled body.

We were able to show by genomic in situ hybridization that the U2 gene clusters are preferentially found adjacent to coiled bodies in the cells we used and that they colocalize with domains enriched in PTFγ, TBP, and hypophosphorylated RNA polymerase II. Although various studies have confirmed that coiled bodies are devoid of newly synthesized RNA (Moreno Diaz de la Espina *et al.*, 1982; Raška, 1995; Schul *et al.*, 1996; Jordan *et al.*, 1997), an indication that RNA synthesis does take place adjacent to coiled bodies was obtained from labeling nascent RNA with the nucleotide analog BrUTP. This approach showed that transcription takes place at the periphery of the coiled bodies overlapping the irregularly shaped domains enriched in factors necessary for snRNA transcription. This association of transcription sites with the periphery of coiled bodies is comparable to the distribution of hyperphosphorylated RNA polymerase II dots, in agreement with the report that these patterns largely colocalize (Grande *et al.*, 1997). Our data suggest that coiled bodies are not simply inactive storage sites but can function as distribution centers from which surrounding genes can be efficiently supplied with transcription factors and RNA polymerase. It should be noted, however, that direct evidence for the expression of the snRNA genes at the coiled body is still lacking. The BrU-labeled newly synthesized RNA found inside the irregularly shaped domains may have been produced by other genes in the vicinity of the coiled body and the RNU2 gene locus. Unfortu-

nately, there has been no reliable method to specifically locate sites of snRNA synthesis in the nucleus. Subsequently, it is unclear whether the snRNA genes that are not associated with a coiled body are active. Since TBP and PTFγ are not only found at the coiled body but also in lower concentrations throughout the nucleus, we cannot rule out the possibility that the unassociated snRNA genes are also being transcribed, possibly at low levels.

Nonetheless, the concentration of the snRNA gene-specific transcription factor PTFγ in domains overlapping with coiled bodies and RNU2 gene loci does suggest a specific functional relationship between these nuclear constituents. We like to propose a model in which the coiled body forms the center around which snRNA genes are organized and where the genes are efficiently supplied with the factors necessary for transcription. Smith *et al.* (1995) have already shown that both U1 and U2 snRNA genes can be grouped around a single coiled body. In our model, the coiled body coordinates the distribution of transcription factors and RNA polymerase to these surrounding genes (Figure 5). This concept is derived from a model we presented earlier in which we proposed that coiled bodies can supply RNA 3′-processing factors to adjacent genes. We showed that RNA 3′-cleavage factors are concentrated in coiled bodies from which they can associate with a nearby active gene. This was based on the observations that RNA 3′-cleavage factors outside coiled bodies were found concentrated in specific domains, called cleavage bodies, which overlapped with an active gene but which relocated to the coiled body after inhibition of transcription (Schul *et al.*, 1996). Cleavage bodies do not overlap with the U1 or U2 snRNA gene (our unpublished data) and therefore do probably not play a role in the transcription and maturation of snRNA, but are more likely to be involved in the processing of RNA from other genes associated with the coiled body.

Since coiled bodies contain high concentrations of U1 and U2 snRNA (Carmo-Fonseca *et al.*, 1991, 1992) it is tempting to speculate that transcripts from the U1 and U2 snRNA gene clusters accumulate in the associated coiled bodies. The coiled bodies could have a function in the processing or transport of the snRNA. Bohmann *et al.* (1995b) already proposed that coiled bodies may play a role in the posttranscriptional modification of snRNA transcripts. Similarly, Smith *et al.* (1995) have suggested that the U2 snRNA gene may associate with the coiled body for the purpose of processing the U2 snRNA. The coiled body could therefore not only play a role in the transcription of the snRNA genes, but also in the maturation of the snRNA transcripts. There are indications, however, that the snRNAs in coiled bodies are not primary transcripts, but mature snRNAs complexed in snRNP particles. SnRNA transcripts are thought to be exported to the cytoplasm to receive a 5′-trimethyl cap after which they are reimported into the nucleus (Mattaj, 1986; Zieve *et al.*, 1988). Several investigators have reported that coiled bodies are labeled by antibodies against the trimethyl cap of mature snRNA, indicating that coiled bodies contain mature snRNA (Carmo-Fonseca *et al.*, 1991; Raška *et al.*, 1991; Matera and Ward, 1993). This does not rule out the possibility, however, that coiled bodies contain primary snRNA transcripts. It is possible that the U1 and U2 snRNAs in coiled bodies are primary transcripts and that the trimethyl cap labeling comes from other mature snRNAs such as U3, U7, and U11, which are also concentrated in coiled bodies (Jiménez-Garcia *et al.*, 1994; Wu and Gall, 1993; Frey and Matera, 1995; Matera and Ward, 1993). Additionally, there are indications that snRNAs can be trimethylated in the nucleus without being exported to the cytoplasm (Terns and Dahlberg, 1994). We would like to emphasize, however, that there is no direct evidence supporting the idea that transcripts produced from the snRNA genes are delivered to the adjacent coiled bodies, although we feel this cannot be ruled out based on the available data.

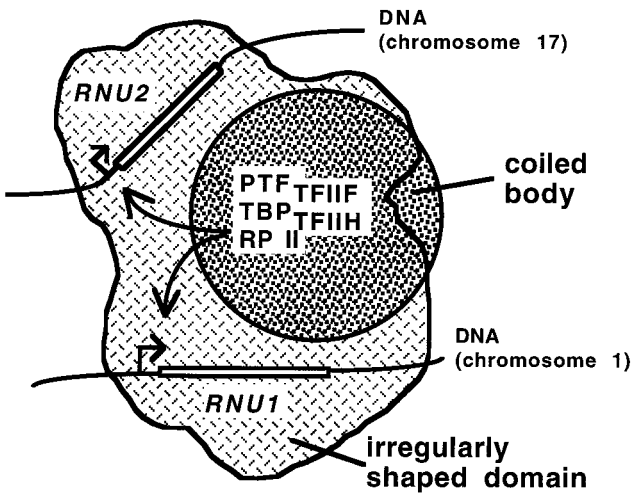

Figure 5. This cartoon represents a model in which the genes for U1 and U2 snRNA (RNU1 and RNU2) are organized around a coiled body. Transcription factors and RNA polymerase II (RPII) are distributed from the coiled body to the associated genes to facilitate and regulate the expression of these genes. The factors are thus concentrated in an irregularly shaped domain partially overlapping the genes and the coiled body. The coiled body itself does not contain DNA or newly synthesized RNA.

An increasing number of studies show that factors involved in transcription or maturation of RNA are enriched in domains associated with coiled bodies. Frey and Matera (1995) showed in human cells that U7 snRNA, necessary for histone mRNA 3′-processing, is concentrated in coiled bodies, while the histone genes are found adjacent to it. Yannoni and White (1997)

have demonstrated that the neuronal protein ELAV from *Drosophila*, involved in RNA splicing, is found associated with coiled bodies, concentrated in dots and irregularly shaped domains (referred to as the ELAV-web). Furthermore, Ishov and Maul (1996) reported that Hep-21 cells contain about one to six foci enriched in the transcription factor NF-1, which are mostly found adjacent to, or partially overlapping, a coiled body. Our model suggests that specific genes, probably a small group of unusual genes that require specific transcription and processing conditions, such as the snRNA genes and histone genes, can associate with the coiled body. There they are efficiently supplied with the transcription factors and/or RNA processing factors they require for gene expression. We like to emphasize that our model neither addresses the dynamic properties of the coiled body nor the relationship between coiled bodies and the nucleolus (discussed in Malatesta *et al.*, 1994). Coiled bodies can have several functions of which a role in the expression of certain genes may be only one.

Efficient transcription of the snRNA genes not only requires activation of the PSE promotor but also activation of the DSE region more upstream of the gene. The transcription factors Oct-1 and Sp1 have been shown to bind to the DSE and can stimulate snRNA gene expression (Ares *et al.*, 1987; Janson and Pettersson, 1990; Yang *et al.*, 1991; Murphy *et al.*, 1992; Tanaka *et al.*, 1992). Interestingly, it has been reported that Sp1 is found in M15 mouse cells in small foci in the nucleus (Larsson *et al.*, 1995). Oct-1 is present throughout the nucleus and concentrated in a large domain often close to the nucleolus in about 30% of the cells (Grande *et al.*, 1997). This domain resembles the brightly labeled perinucleolar domains we occasionally observed for PTFγ. Pombo *et al.* (1998) have recently found that this perinucleolar PTF domain colocalizes with the Oct-1 domain but is not associated with the snRNA genes. Further studies will have to elucidate the spatial organization of these transcription factors in relation to the snRNA genes at the coiled body.

There is an emerging view that nuclear domains enriched in transcription factors and RNA processing factors can be spatially associated with specific genes. A well known example of this organizational principle is the nucleolus in which ribosomal genes are grouped in a nuclear structure together with the factors required for rRNA synthesis and maturation. Our findings indicate that a similar organizational principle may underlie the role of the coiled body in the synthesis of snRNA. This suggests that the association of specific genes with domains enriched in transcription factors and RNA processing factors may be a common mechanism of higher order gene regulation in the eukaryotic cell.

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