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U2 and U1 snRNA Gene Loci Associate With Coiled Bodies

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Abstract The coiled bodies are nuclear structures rich in a variety of nuclear and nucleolar components including snRNAs. We have investigated the possibility that coiled bodies may associate with snRNA genes and report here that there is a high degree of association between U2 and U1 genes with a subset of coiled bodies. As investigated in human HeLa cells grown in monolayer culture, about 75% of nuclei had at least one U2 gene associated with a coiled body, and 45% had at least one U1 locus associated. In another suspension-grown HeLa cell strain, 92% of cells showed association of one or more U2 genes with coiled bodies. In contrast to the U2 and U1 gene associations, a locus closely linked to the U2 gene cluster appeared associated with a coiled body only in 10% of cells. Associated snRNA gene signals were repeatedly positioned at the edge of the coiled body. Thus, this association was highly nonrandom and spatially precise. Our analysis revealed a much higher frequency of association for closely spaced "doublet" U2 gene signals, with over 80% of paired signals associated as opposed to 35% for single U2 signals. This finding, coupled with the fact that not all genes were associated in all cells, suggested the possibility of a cell-cycle-dependent, possibly S-phase, association. However, an analysis of S- and non-S-phase cells using BrdU incorporation or cell synchronization did not indicate an increased level of association in S-phase. These and other results suggested that a substantial fraction of paired U2 signals represented association of U2 genes on homologous chromosomes rather than only replicated DNA. Furthermore, triple label analysis showed that in a significant fraction of cells U1 and U2 genes were both associated with the same coiled body. U1 and U2 genes were closely paired in approximately 20% of cells, over 60% of which were associated with a readily identifiable coiled body. This finding raises the possibility that multiple genes of a particular class may be in association with each coiled body. Thus, the coiled body may be a dynamic structure which transiently interacts with or is formed by one or more specific genetic loci, possibly carrying out some function related to their expression.

Key words: nuclear bodies, coilin, in situ hybridization, HeLa cells, snRNA

Coiled bodies are small, spherical, subnuclear structures, generally numbering one to five per nucleus, which display remarkably similar morphology in animal and plant cells [reviewed in Brash and Ochs, 1992; Lamond and Carmo-Fonseca, 1993]. Found primarily in highly proliferative cells such as cancer cells, the function of coiled bodies remains a mystery, but their striking evolutionary conservation indicates some crucial role in the nucleus. The size and number of coiled bodies has been shown to fluctuate during the cell cycle, becoming larger and less numerous as the cell progresses from early G1 through S-phase [Andrade et al., 1993]. Furthermore, coiled bodies have been shown to respond to environmental stimuli such as hormone stimulation [Ochs et al., 1995] and temperature [Carmo-Fonseca et al., 1993]. These results indicate that coiled bodies are dynamic subnuclear structures.

In recent years, heightened interest in coiled bodies has resulted from studies which have defined some of their molecular components. A variety of nucleolar and nucleoplasmic constituents are found concentrated in coiled bodies. The presence of splicing components, including snRNP antigens [Carmo-Fonseca et al., 1992; Fakan et al., 1984; Huang and Spector, 1992] and snRNAs of the pre-mRNA splicing class

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[Carmo-Fonseca et al., 1991a,b; Matera and Ward, 1993] and non-snRNP splicing factor U2AF [Carmo-Fonseca et al., 1991b; Zamore and Green, 1991; Zhang et al., 1992], opens the possibility that coiled bodies may be involved in splicesome assembly or pre-mRNA splicing. However, this possibility is not supported by the finding that SC35, a factor thought to be essential for splicesome assembly [Fu and Maniatis, 1990], is among several nuclear proteins that are not detected in coiled bodies [Raska et al., 1991; Spector et al., 1991]. The sphere organelles found in amphibian germinal vesicles have many properties in common with coiled bodies [reviewed in Gall et al., 1995]. The association of the sphere organelle with histone gene loci in amphibians opens the possibility that these structures may have a role in the processing of some classes of RNA [Gall et al., 1981; Callan and Gall, 1991]. Nevertheless, the function of coiled bodies remains elusive.

Among the numerous factors found in coiled bodies is the phosphoprotein p-80 coilin [Andrade et al., 1991]. This factor, the function of which is unknown, exists almost exclusively in coiled bodies, though recent EM studies have located it in other regions of the nucleus [Puvion-Dutilleul et al., 1995]. Antibodies to this protein have greatly aided the use of immunofluorescence in the study of this structure. There is an extensive variety of snRNAs within coiled bodies, including U2, U4/U6, and U5 [Carmo-Fonseca et al., 1991b, 1992; Huang and Spector, 1992]. U1 is also present in coiled bodies but is also diffusely distributed throughout the nucleus [Carmo-Fonseca et al., 1991a,b, 1992]. Detection of the snRNA trimethylguanosine cap structure in coiled bodies has suggested that these are mature snRNAs [Carmo-Fonseca et al., 1991b; Raska et al., 1991]. This, however, does not rule out the possibility that pre-snRNAs could be present.

The coiled body is clearly a novel nuclear compartment. Recent advances in the understanding of nuclear architecture have led to an emerging view of the nucleus as structurally and functionally compartmentalized [e.g., see Moen et al., 1995]. The nucleolus, devoted to rRNA metabolism, is the prototype for subnuclear compartmentalization and establishes a precedent for the possibility that genes on different chromosomes can specifically associate to facilitate a common purpose, such as the production of a specific class of RNA. However, other functionally undefined compartments enriched in RNA processing components, such as coiled bodies, have been generally thought of as having no specific spatial relationship to the DNA compartment. We previously proposed [Xing et al., 1993] that an integrated structural organization may exist between the splicing factor–rich compartment [Nyman et al., 1986; Spector et al., 1991] marked by high levels of SC-35 [Fu and Maniatis, 1990] and poly (A) RNA [Carter et al., 1991, 1993]. Recent evidence further supports the idea of a nonrandom organization of specific genetic loci relative to these SC-35 domains (Xing et al., in press; Clemson et al., in press; Coleman and Moen, unpublished). Here we investigate the question of whether the coiled body "compartment" shows any specific relationship to genetic loci, specifically U1 and U2. This question is prompted to some extent by the high concentration of U2 and, to a lesser extent, U1 in the coiled body. Other work has found that the highest concentration of several pre-mRNAs is at or near their site of transcription [reviewed in Xing and Lawrence, 1993]. Further, the snRNAs represent a class of nonpolyadenylated pol II transcripts that may have unique processing requirements that could conceivably involve components of the coiled body. Hence, we speculated that coiled bodies, rich in a variety of snRNAs and other diverse RNA processing components, may associate with snRNA gene loci.

Using fluorescence in situ hybridization, we report here the association of coiled bodies, as defined by an antibody to p80 coilin, with a very substantial fraction of, but not all, U2 genes (Fig. 1). This association is seen for both adherent and suspension HeLa cells. This association is also found for the U1 gene locus, although to a lesser extent. We further report the preferential association of paired gene signals with coiled bodies. Such signals could represent either replicated DNA or paired alleles, although our investigations find no S-phase–related increase in association of snRNA genes and coiled bodies. A substantial fraction of doublet signals represent pairing of alleles from homologous chromosomes. Furthermore, the association of heterologous U1/U2 locus pairs with coiled bodies was also seen. The significant level of association of these homologous and heterologous gene pairs leads us to propose that individual coiled bodies may be associated with or be formed by multiple snRNA genes.

METHODS

Cell Culture and Fixation

Monolayer HeLa S3 cells were grown on coverslips in DMEM plus 10% fetal calf serum with 10 μg/ml gentamicin. Prior to fixation, cells were washed first at room temperature and then on ice in Hanks balanced salt solution (HBSS) (Gibco/BRL, Gaithersburg, MD), followed by successive washes on ice in cytoskeletal (CSK) buffer (100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 10 mM PIPES, pH 6.8 [Fey et al., 1986]) prior to a 0.5–5.0 min extraction in CSK buffer containing 0.5% Triton X-100 plus 2 mM Vanadyl ribonucleoside complex (BRL). Cells were then immediately fixed in 4% paraformaldehyde in 1× PBS (pH 7.4) for 10 min and stored at 4°C in 70% EtOH. Alternatively, a suspension HeLa cell strain was obtained form J. Stein (University of Massachusetts Medical Center) and grown in media described above in spinner flasks and plated onto coverslips 1.5–2 h prior to extraction and fixation.

S-Phase Analysis

Cells in S-phase were detected by incubation of HeLa cells for 30 min with bromodeoxyuridine (BrdU) followed by immunofluorescence with anti-BrdU antibody as previously described [Carter et al., 1991].

S-phase synchronized cells, kindly provided by F. Aziz (Stein Lab, University of Massachusetts), were prepared from suspension HeLa cells blocked at G1/S by treatment with methotrexate. Cells were plated on coverslips 4 h after release from block and extracted and fixed 2 h later.

Antibodies and DNA Probes

The U2 locus contains numerous copies of a tandem repeat [Van Arsdell and Weiner, 1984]. To detect this locus, we used a 6 kb genomic probe, pTP18, provided by John Bodnar and originally cloned in the Weiner Lab. The U1 gene locus was detected using a 13 kb probe provided by James Dahlberg (University of Wisconsin, Madison, WI) [Lund and Dahlberg, 1984]. The chromosome 17 centromere probe p3.6 [Barker et al., 1987] and the chromosome 17 2-20 locus probe were gifts from Ray White (University of Utah Medical Center, Salt Lake City, UT) and Mark Skolnick (University of Utah Medical Center, Salt Lake City, UT), respectively.

A rabbit polyclonal antibody (R288) against the coiled body factor p80 coilin [Andrade et al., 1991], kindly provided by Edward Chan (The Scripps Research Institute, La Jolla, CA), was used to delineate coiled bodies. An anti-PCNA antibody was the gift of Linda Malkas (University of Maryland, Baltimore, MD).

Fluorescent In Situ Hybridization and Immunofluorescent Staining

For each coverslip, 50 ng of nick-translated DNA, incorporated with either biotin-16-dUTP or digoxigenin-11-dUTP, was lyophilized with 10 μg each of sonicated salmon sperm DNA, *E. coli* tRNA, and human Cot I DNA (BRL). The probes were resuspended in 100% formamide prior to heat denaturation for 10 min at 70°C. A more detailed protocol can be found elsewhere [Johnson et al., 1991]. Briefly, for hybridization to DNA, cells were heat-denatured in 70% formamide, 2× SSC at 70°C for 2 min or by incubation in 0.07M NaOH, 70% EtOH for 5 min to remove RNA. All hybridizations were incubated for 3 h to overnight at 37°C.

Antibody staining was carried out in antibody diluted 1:500 in 4× SSC, 1% BSA for 1.5 h. Three washes (4× SSC, 4× SSC/.1% Triton X-100, 4× SSC) of 15 min each were then done. The antibody was detected using a fluorescein, rhodamine, or AMCA tagged secondary (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:500 in 4× SSC. Washes were done as before. Ordinarily, in experiments using both hybridization and immunofluorescence, the coilin staining was done first and the cells were fixed in 4% paraformaldehyde since hybridization resulted in a reduction of coilin staining.

Microscopy and Image Analysis

A Zeiss (Oberkochen, Germany) Axioplan microscope equipped with a multibandpass epifluorescence filter (Chroma, Brattleboro, VT) was used for analysis. All images were captured with a Photometrics series 200 CCD camera using a high resolution, shallow depth-of-field objective (NA = 1.4) (Zeiss). The analysis of gene position relative to coiled bodies was done by first viewing the target signal under a single filter set to verify the efficiency of hybridization and then switching to a separate filter set to verify the efficiency of the coilin staining prior to viewing simultaneously. This protocol assured that the selection of cells to be scored was random with respect to whether there was any association of

the gene signal and coiled body. For each experiment, at least two investigators scored the slide separately.

RESULTS

Coiled Bodies Associate With U2 Gene Loci

We investigated the position of the human U2 gene complex relative to coiled bodies within HeLa cell nuclei using simultaneous fluorescent in situ hybridization and immunofluorescence with an antibody against the coiled body–specific protein, p-80 coilin [Andrade et al., 1991]. The U2 locus resides on chromosome 17 as a tandem array of 10–20 units of 6 kb each [Van Arsdell and Weiner, 1984]. The HeLa cells used are trisomic for chromosome 17. On average, the HeLa cells contained three coiled bodies (Table I). During several experiments, using conditions which remove RNA from the cell and allow hybridization to DNA (see Methods), we consistently found that approximately 75% of cells had at least one U2 gene signal in contact with a coiled body (Figs. 1A, 2A). These signals which appeared to be in contact with a coiled body were termed *associated signals*. Greater than 40% of cells generally had more than one locus associated with one or more coiled bodies, and 10% of cells (Figs. 1A, 2A) had at least three gene signals associated with a coiled body (some cells up to six signals). The graph in Figure 2B illustrates the percentage of gene signals which are associated with coiled bodies, with approximately 40% of U2 loci associated. It should be noted that these numbers may be an underestimate since very small concentrations of coilin may escape detection in these assays. Because signals from coiled bodies and the U2 gene complex occupy such a small percentage of nuclear volume ($<1\%$), the chance of random overlap between these signals is minuscule; however, to test the specificity of association between the U2 genes and the coiled body we did similar analyses using two other sequences on chromosome 17. Initially, p3.6, a chromosome 17 centromeric repeat, was used. To more rigorously assess the specificity of the U2 gene/coiled body association, a probe named 2-20 was obtained (see Methods). Using a previously described method [Lawrence et al., 1990], we mapped this probe to a region of chromosome 17 less than 0.5 microns (approximately 500 kb) from the U2 gene locus in interphase nuclei (data not shown). Signals from 2-20 and from the relatively large block of the p3.6 repeated sequence were often positioned close to a coiled body, appearing to make contact in about 10–15% of cells (Fig. 2A). In most cases, even these associated signals did not show the intimate juxtaposition with the coiled body that was typical of the U2 signals. Overall, only about 5% of signals using these probes appeared to make any contact with coiled bodies (Fig. 2B). Hence, the high degree of association and the intimate spatial relationship between coiled bodies and U2 gene loci was not seen for sequences closely linked to the U2 gene.

An earlier report using suspension HeLa cells found that snRNA genes were not associated with coiled bodies [Matera and Ward, 1993] (see Discussion). In order to determine if this reported lack of association was a phenomenon of suspension HeLa cells, we tested a suspension HeLa cell strain for coiled body associations to U2 loci. These suspension cells had, on average, 2.7 coiled bodies per nucleus. The percentage of cells with at least one coiled body/U2 association was even higher than for the adherent cells, with 92% having at least one gene associated (Table I). This result indicated that coiled body associations were not reduced in HeLa cells grown in suspension culture.

U2 signals were often seen associated with very small coiled bodies (Fig. 1B), suggesting the possibility that the coiled body may actually form at the site of the U2 gene (see Discussion). The morphology of the U2 signal association with a coiled body was quite consistent. There

Fig. 1. Visualization of coiled body associations with snRNA genes. Fluorescent in situ hybridization was used to detect the snRNA genes simultaneously with immunofluorescence with anticoilin staining. Analysis was done in human HeLa cells. **A:** *Coiled bodies (red) spatially associate with U2 gene loci (green)* on chromosome 17 (three in HeLa cells). Clockwise from upper left: two loci associated, one locus associated, and all three loci associated with coiled bodies. **B:** Arrow indicates U2 locus often seen associated with small coiled body (red). **C:** Coiled bodies (green) found to associate with U1 gene loci (red) on chromosome 1 (two in HeLa cells). **D:** Paired U2 gene loci (green) have a high degree of association with coiled bodies (red). Cell has only three U2 signals, so alleles have paired. **E:** Cell showing more than three U2 signals (green), suggesting the presence of replicated DNA. Presumptive replication doublets *are associated with a single coiled body (red).* **F:** *Example of all* three alleles of U2 loci (red) associated with one coiled body (green). **G:** Foci enriched in proliferating cell nuclear antigen (PCNA) (green) do not overlap coiled bodies (red). **H:** Heterologous genes are sometimes observed to pair at a coiled body. U2 locus (green) and U1 locus (red) are seen associated encircling a coiled body (blue). The other two coiled bodies each have a U2 locus associated.

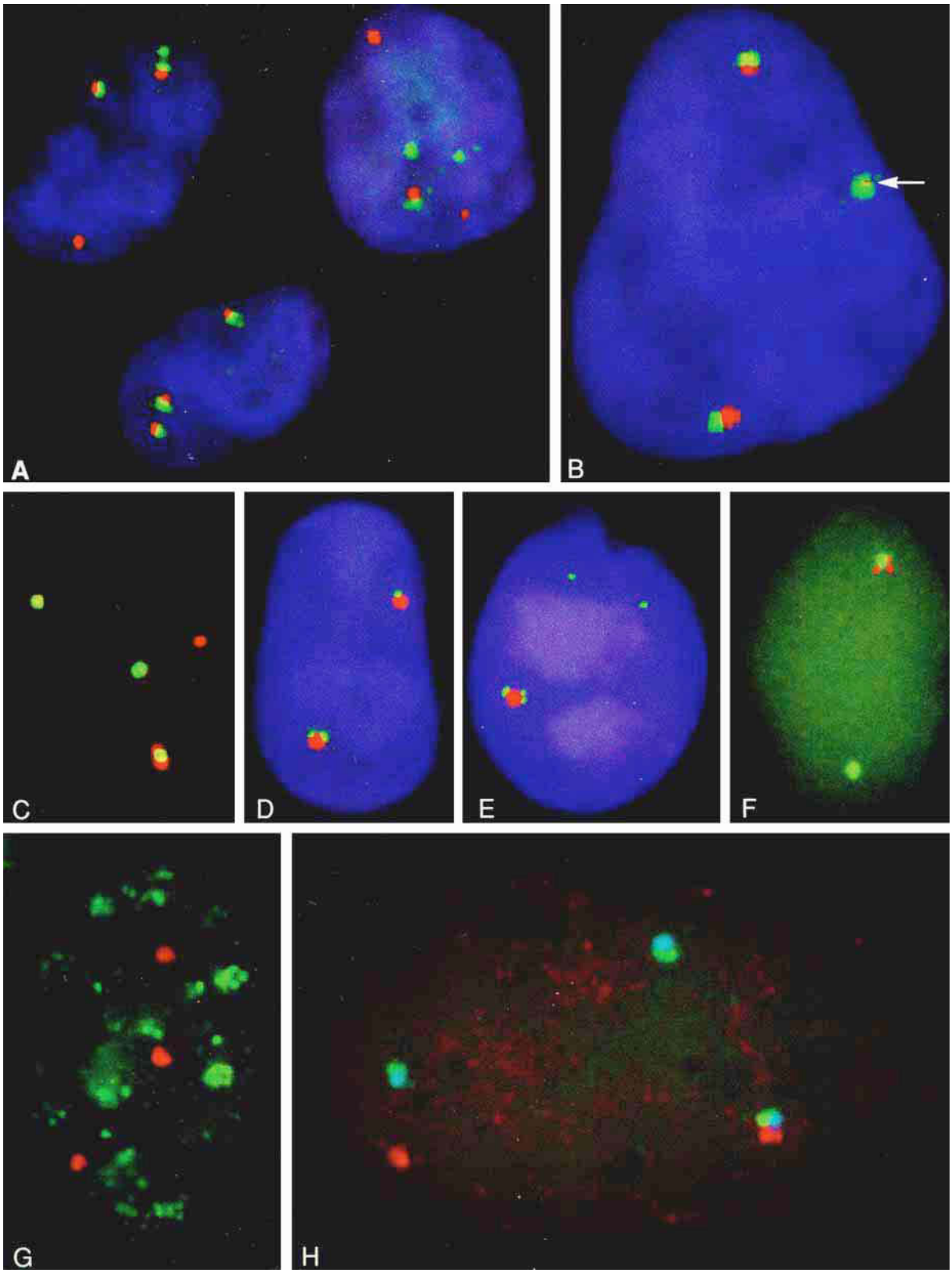

Figure 1.

was never more than one coiled body associated with a U2 signal. Furthermore, the U2 signal was consistently positioned directly at the edge of a given coiled body rather than within it (Fig. 1A,B). This positioning was seen for approximately 90% of associated signals, with the remaining 10% generally being coiled bodies which were too small to distinguish whether the coincident gene signal was within the coiled body or at its edge. Taken together, these data indicate that at least one U2 gene complex allele is not only associated with, but also specifically positioned at the edge of a coiled body in most of these cells.

The U1 Gene Locus Associates With Coiled Bodies

To test whether other snRNA genes are associated with coiled bodies, we investigated the nuclear position of the U1 snRNA gene complex, another tandemly repeated array, on chromosome 1 [Lund and Dahlberg, 1984]. Our HeLa cells are diploid for chromosome 1. As with the U2 genes, a significant but somewhat smaller fraction of cells (45%) had at least one gene signal associated with a coiled body (Figs. 1C, 2A). Expressed as a percentage of gene signals, about 25% of U1 signals were associated with a coiled body (Fig. 2B). As with the U2 genes, U1 signals were specifically positioned at the edge of coiled bodies. As noted in the introduction, U1 RNA is more diffuse in the nucleus than U2. While the reason for the lower level of association of U1 is unknown, it appears that U1 RNA, and the gene, may have a lower affinity for coiled bodies than U2.

Paired Gene Signals More Frequently Associate With Coiled Bodies

It was frequently noted that U2 gene signals would appear closely associated in pairs. Upon careful analysis, it was seen that the probability of association with a coiled body increased dramatically from 35% for single U2 signals to over 80% for "doublet" signals (Fig. 3). Doublet signals were defined as two discrete but very closely spaced fluorescent spots less than 1 micron apart. As illustrated in Figure 1D,E, the vast majority of cases analyzed showed the coiled body to interact with both signals of a pair, often appearing between them. Occasionally, three or more signals would associate with a single coiled body (Fig. 1F). The likelihood that all four small entities (three genes and a coiled body) would localize to the same place within the nucleus by random chance is vanishingly small. Hence, the finding of multiple related gene loci at the coiled body is further evidence of the significance and specificity of this association. The detection of gene doublets could occur either when the locus has replicated or when alleles on separate chromosomes pair. Significant numbers of paired signals were found in cells containing only three U2 gene signals, indicating allelic pairing (Fig. 1D), as well as with greater than three signals (replicating cells) (Fig. 1E). In both cases, these

TABLE I. Comparison of Suspension and Adherent HeLa Cells*

	Adherent HeLa		Suspension HeLa	
	Unsynchronized	+BrdU	Unsynchronized	S-phase
Cells (#)	208	122	100	121
Coiled bodies per cell (#)	3.0	2.7	2.7	1.5
≥ 1 locus associated (%)	76	78	92	80
Signals associated (%)	42	48	63	41
Doublet cells (%)	34	36	39	35
Doublet associated (%)	79	84	87	82

*Comparisons are also made between S-phase cells and random (unsynchronized) populations. For adherent HeLa cells, S-phase cells were identified by bromodeoxyuridine (BrdU) incorporation. For suspension cells a synchronized population in late S-phase was used. Cells, (#) number of cells scored; ≥ 1 locus associated (%), percentage of cells with at least one U2 signal in contact with a coiled body; Signals associated (%), overall percentage of gene signals associated with coiled bodies; Doublet cells (%), percentage of cells in which paired U2 gene signals are evident; Doublet associated (%), percentage of paired signals found to be associated with a coiled body.

Fig. 2. Level of association of different genetic loci with coiled bodies. **A:** Percentage of cells having at least one, two, three, or more of four different loci associated with coiled bodies. The percentage of cells with at least one chromosome 17 centromere scored as associated is likely an overestimate, since signals which made only very marginal contact with the coiled body were included as associated, and the size of the centromeric signal and proximity to the U2 locus also inflate these numbers. **B:** Percentage of total hybridization signals seen to be in contact with a coiled body. The U2 locus, chromosome 17 centromere (Ch. 17 cent.), and locus 2-20 are all on chromosome 17. The U2 locus and 2-20 have been mapped to within 500 kilobases of each other by interphase analysis. The U1 locus is on chromosome 1. n, number of signals scored.

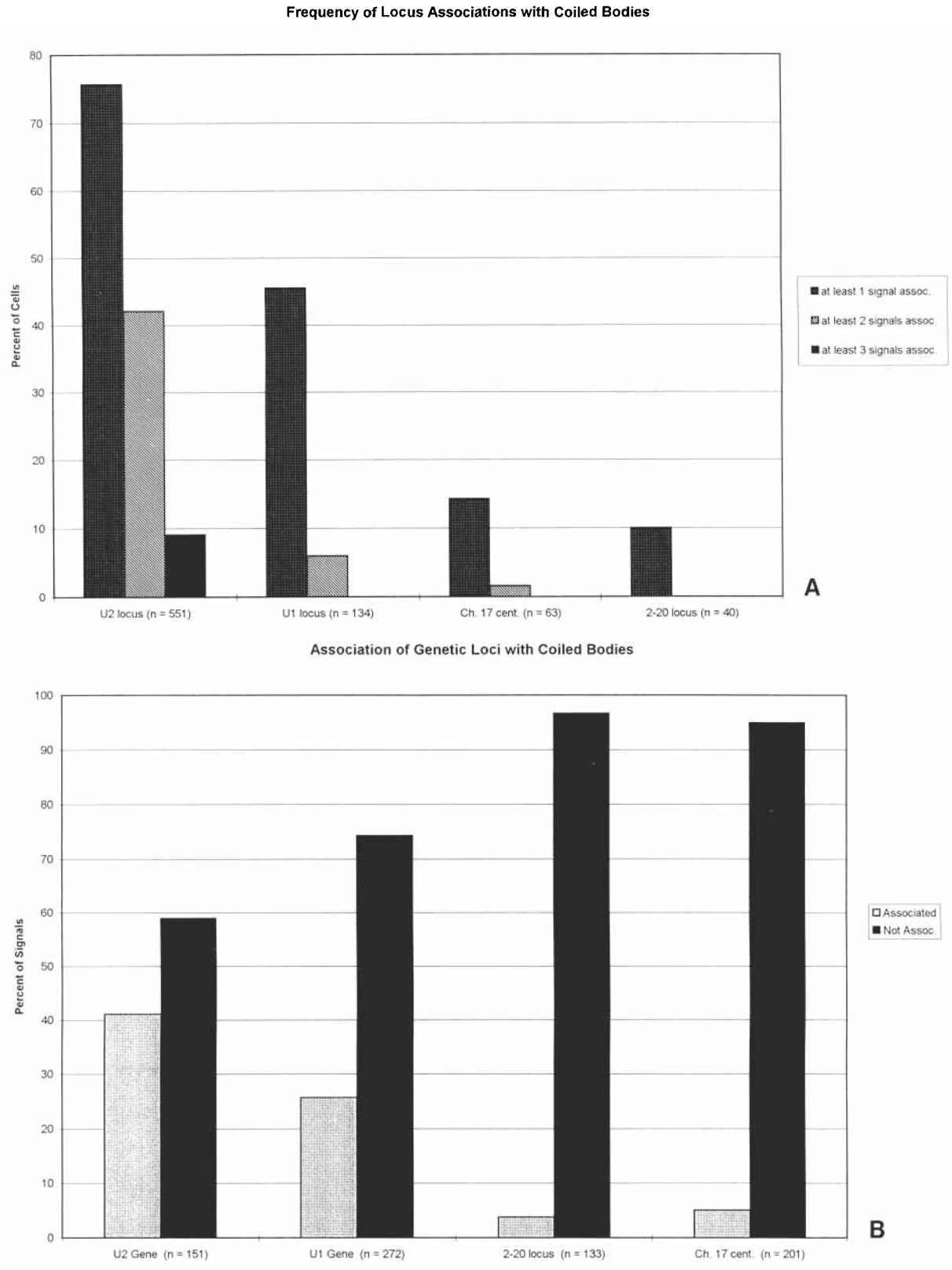
Frequency of Locus Associations with Coiled Bodies
Percent of Cells
80
70
60
50
40
30
20
10
0
U2 locus (n = 551)
U1 locus (n = 134)
Ch. 17 cent. (n = 63)
2-20 locus (n = 40)
at least 1 signal assoc.
at least 2 signals assoc.
at least 3 signals assoc.
A
Association of Genetic Loci with Coiled Bodies
Percent of Signals
100
90
80
70
60
50
40
30
20
10
0
U2 Gene (n = 151)
U1 Gene (n = 272)
2-20 locus (n = 133)
Ch. 17 cent. (n = 201)
Associated
Not Assoc.
B

Figure 2.

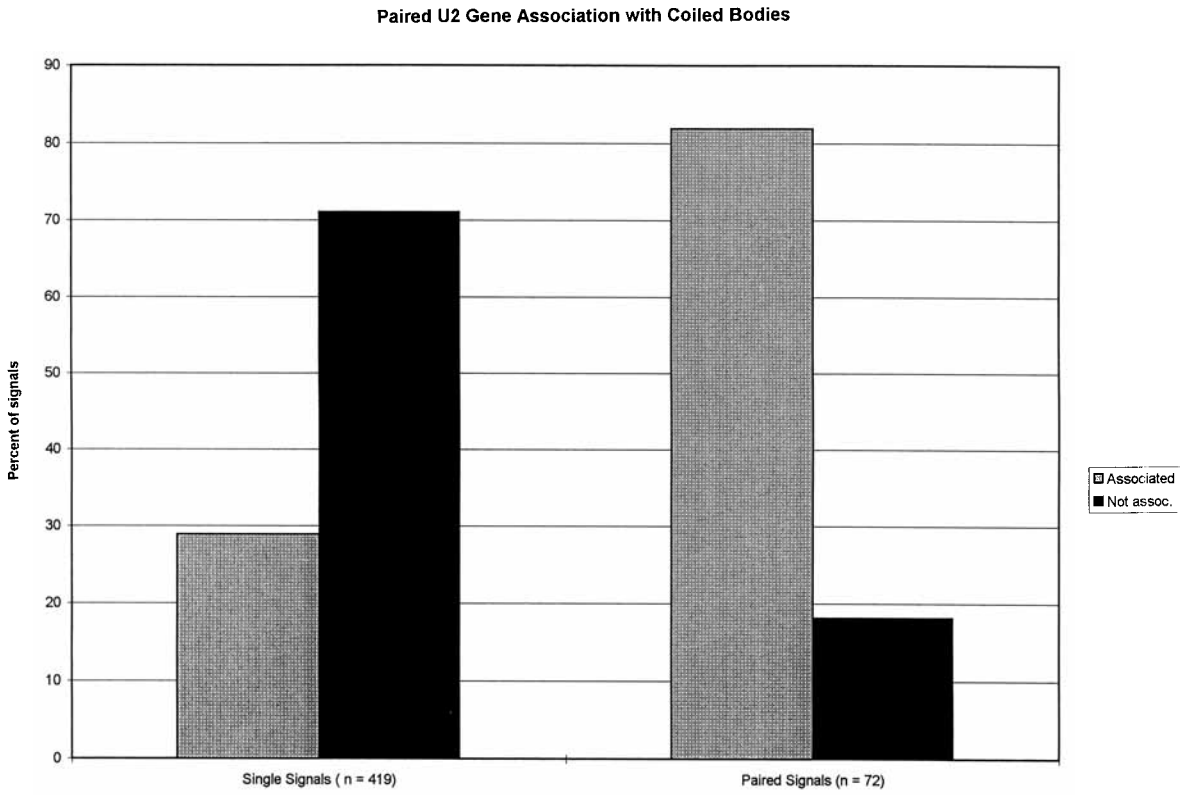

Fig. 3. Difference in level of association with coiled bodies of single U2 gene signals and paired U2 signals. Data show the percentage of single or double hybridization signals found to be in contact with a coiled body. This paired signal analysis did not differentiate between replication pairs and paired alleles. As illustrated in Fig. 1D,E,F,H, signals judged to be paired were generally very close if not touching and were no more than ~1 micron apart. n, number of signals or pairs scored.

paired signals showed a remarkable increase in coiled body associations. Given the potentially important implications of this phenomenon, it was investigated in more detail (see Results below).

S-Phase Associations

The association of coiled bodies with only some U2 gene loci in some cells indicates that this association may be highly dynamic, possibly occurring only at specific times during the cell cycle. Although doublet/coiled body association was often seen in cells in which the number of gene signals was less consistent with replicated DNA (see below, this section), such doublets still suggested the possibility of coiled body associations with snRNA genes in replicating cells. To investigate whether the variations seen are cell-cycle-related, we developed an approach by which the cell cycle stage of individual cells could be detected in S-phase using incorporation of bromodeoxyuridine (BrdU). Our cell cycle analysis of coiled bodies found that the number of detectable coiled bodies per cell was greatest in early G1 (sometimes greater than ten per cell) and then decreased during late G1, S, and G2 [also described in Andrade et al., 1993]. The level of association of U2 signals with coiled bodies was not found to be significantly higher in S-phase cells than in non–S-phase cells (Table I). However, a slight increase in the number of cells with more than one signal associated did increase slightly (not shown). It should be noted that, although cells with greater than three U2 gene signals were more prevalent in the BrdU + cells, they still did not represent a major fraction of the cells assayed and paired signals were not substantially more common than in the BrdU – population, which includes G2 cells (Table I).

It has been reported that structures corresponding to sites of DNA replication, termed *replication factories,* are a subset of nuclear bodies [Hozak et al., 1994]. It was conjectured that coiled bodies may colocalize with replication factories found in proliferating cells, with the snRNA gene loci serving as some type of control point for replication. However, immunofluorescence studies saw no overlap between

coilin and distinct regions rich in proliferating cell nuclear antigen (PCNA), a component of these replication factories (Fig. 1G). Thus, it would appear that coiled bodies and prominent nuclear bodies enriched in PCNA are distinct structures.

Another method was employed to investigate U2/coiled body associations in S-phase cells. A suspension HeLa cell culture (see Methods and Results above) was used to obtain a population of cells synchronized in late S-phase by methotrexate block. Cells at this stage had fewer coiled bodies, averaging 1.5 per nucleus, than the unsynchronized culture. An analysis of U2 gene locus association with coiled bodies revealed that the level of gene association with a coiled body was actually slightly lower in these synchronized cells (Table I), with 80% of cells having at least one gene associated vs. 92% of the cells in the unsynchronized population. This drop in associations could be due to the 50% drop in the average number of coiled bodies per nucleus in the late S-phase cells. In fact, given the drop in the number of coiled bodies, the probability that a given coiled body has a gene associated with it does increase in these S-phase cells, despite the fact that the percent of genes associated with a coiled body does not.

Using two different methods, we could determine no significant increase in the percentage of U2 loci associated with coiled bodies in S-phase cells. Although not an exhaustive study of the cell cycle, these studies suggest that the paired signal associations we noted were possibly not a cell cycle phenomenon. So, we decided to further investigate nonreplicative gene pairing.

Homologous and Heterologous Pairing of snRNA Loci

The HeLa cells studied contained three copies of chromosome 17 and, thus, three copies of the locus containing the tandem U2 gene cluster. The vast majority of interphase nuclei showed three strong, discrete hybridization signals, indicating a high efficiency of hybridization. Careful examination of the number of gene signals in cells containing pairs or doublets showed that in many or most cases the number of signals remained at three (Fig. 1D), although cells showing four or more signals were also observed (Fig. 1E). Results such as those illustrated in Figure 1D, where only three signals are present, indicates that in many cells the paired signals represented close association of homologous alleles on separate chromosomes rather than replicated DNA from sister chromatids of a single chromosome. As indicated in Table II, the frequency of cells with U2 gene pairs was significant (20%), and 80% of these pairs were associated with a visible coiled body. In a study of the U1 gene locus, about 14% of cells contained U1 signal pairs, and 60% of these were associated (Table II). This lower level of association for U1 pairs may be expected given the lower overall association of U1 genes with coiled bodies (25%) (Fig. 2B). Nonetheless, this association with pairs of genes would seem to be true of U1 as well as U2.

These results raised the important question whether or not heterologous pairs of genes could be found and if these pairs associated with coiled bodies. The use of simultaneous detection of U1, U2, and coiled bodies (Fig. 1H) allowed us to determine that the association of U1 and U2 gene loci occurred in approximately 20% of close to 400 cells studied. The gene signals were in most cases immediately juxtaposed, as shown in Figure 1H. We determined that these heterologous pairs were associated with coiled bodies over 60% of the time (Table II). Given the very tiny volume each gene signal occupies, we interpret their association with each other and with the coiled body to be highly nonrandom. In fact, the percentage of cells containing some form of pairing, either homologous or heterologous, involving U1 and/or U2 loci is approximately 52%. These results suggest that a single coiled body frequently associates with multiple snRNA loci, perhaps mediating some gene pairing, in order to carry out some function common to these genes.

DISCUSSION

Results presented here demonstrate a spatial association of two snRNA gene loci, U1 and U2, with coiled bodies. In HeLa cell nuclei, we found

TABLE II. Comparison of the Frequency of Paired Gene Signals in HeLa Cells*

	Paired Signals		
	U1/U1	U2/U2	U1/U2
Cells scored	353	350	396
Cells with pairs (%)	14	22	20
Pairs with coiled bodies (%)	62	80	63

*The paired signal analysis does not differentiate between replication pairs and paired alleles. Signals were judged to be paired if they were less than ~1 micron apart.

the frequency of association with coiled bodies to be much greater for U1 and U2 genes than for another locus located very close to the U2 locus. Furthermore, associated snRNA gene loci showed a highly precise and consistent spatial relationship with the coiled body, with the DNA signal localized at the outer border of the coiled body, directly abutting it but only rarely appearing to lie within it. For two different strains of HeLa cells, the majority of cells (75–90%) showed at least one U2 locus associated with a coiled body. However, not all gene signals localized with coiled bodies, and not all coiled bodies localized with gene signals. Overall, 40% of all U2 gene signals and 25% of U1 signals associated with a coiled body. U2 gene signals which appeared as doublets had a markedly higher probability of association, with 80–85% of U2-U2 doublets associated with a coiled body. Doublets could indicate the presence of replicated DNA, and, since the frequency of association suggests a potentially transitory or dynamic association (see below), cell cycle analysis was done, primarily with respect to S-phase. U2–coiled body association was not restricted to a particular phase of the cell cycle, and only a slight increase in association was noted at S-phase in a bromodeoxyuridine incorporation experiment. Further analysis indicated that many doublet signals appeared to represent allelic pairing of one U2 locus with another, at the coiled body, rather than replication of a single locus. Moreover, in a substantial fraction of cells, U1 and U2 gene loci were paired and associated with the same coiled body.

An important aspect of our results is that we find substantial association of multiple snRNA genes with a single coiled body. Given that we assessed only two snRNA genes, it is possible that if all the implicated genes (including numerous snRNAs as well as possibly histone genes [Frey and Matera, 1995]) could be simultaneously visualized in a single cell, all coiled bodies would have one, and probably multiple, specific genes associated with them. We found that U1 and U2 associate with each other in approximately 20% of the cells and approximately 52% of cells showed either homologous or heterologous pairing involving U1 and/or U2 loci. When one considers that in the cells studies there were most often several coiled bodies (average of three per cell) with which the genes could associate and that we were only visualizing two of the snRNA genes, the frequency of association is likely significant. This is best illustrated by comparison with the nucleolus, where genes from several different chromosomes associate. In a cell type with four nucleoli, we estimate the probability that rRNA genes from two nonhomologous chromosomes are together in the same nucleolus is only 25%. In this respect the association of multiple snRNA genes at the coiled body may be analogous to the nucleolus, but with several notable differences: nucleoli are readily visible in all cell types, the structural relationship of rRNA genes with the nucleolus is thought to be more constant, and rRNA genes reside within the nucleolus, whereas snRNA genes localize to the outer border of the coiled bodies. Unlike the rRNA genes and the nucleolus, the functional significance of the snRNA gene association with the coiled body remains to be determined.

In our view, the spatial relationship of these specific loci with coiled bodies is not merely a coincidence of genomic/nuclear organization but is likely related to the fact that the snRNA genes encode a specific class of RNAs, and the coiled body contains high concentrations of these same RNAs as well as processing components for various classes of RNA. However, the relationship could be related to any of several different aspects of snRNA gene function, including transcription, autoregulation of snRNA gene expression, processing of the snRNAs, delivery of the snRNAs into the coiled body for transport, activity in processing of other RNAs, or snRNP assembly. While it may appear an obvious possibility, two observations suggest that the situation is likely more complex than simply the transcription and delivery into the coiled body of U2 snRNA. First, U2 snRNA within the coiled body is known to have a mature trimethylguanosine cap structure [Carmo-Fonseca et al., 1991b; Raska et al., 1991]. Furthermore, evidence indicates that the processing of the RNA and addition of the cap structure occurs in the cytoplasm [Zieve et al., 1988]. Hence, these results do not fit with the idea that the snRNA would go directly from the gene into the coiled body. The fact that high levels of ^{3}H-uridine do not incorporate into coiled bodies would also appear to argue against this, although there are limitations to the interpretation of such experiments [discussed in Moen et al., 1995].

Another possibility is that the location of some snRNAs with coiled bodies is related to the processing of the snRNA itself. What makes this

possibility more plausible in our view is the prior finding of a relationship of histone gene loci to sphere organelles in *X. laevis,* which contain U7 RNA known to be involved in the processing of histone RNAs [reviewed in Gall et al., 1995]. The sphere organelles are in many respects analogous to coiled bodies, and the relationship of histone loci with U7 containing coiled bodies has recently been demonstrated in human cells [Frey and Matera, 1995]. Since U7 RNA functions in processing of histone RNA, it is highly likely that the gene's interaction with the coiled body may facilitate histone RNA processing. By analogy, the U2 gene may associate with coiled bodies for the purposes of processing the U2 snRNA. While some final processing is known to occur in the cytoplasm, as noted above, the U2 snRNA genes are organized as very large clusters that may possibly produce complex primary transcripts which would require extensive processing prior to their exit to the cytoplasm (Smith, unpublished results). Therefore, if the U2 genes associated with the coiled body are transcriptionally active, the coiled body may contain components necessary for the posttranscriptional processing of these RNAs. Interestingly, snRNAs are more similar to histone RNAs than to other mRNAs, in that histone RNAs are also small, nonpolyadenylated RNAs that do not contain introns and are transcribed from clustered genes carrying large segments of noncoding interspersed sequences. The ribosomal RNA genes also have similar properties, and the coiled body is frequently seen to associate with the nucleolus [Raska et al., 1990; Ochs et al., 1994]. However, there is, as yet, no functional evidence to suggest that coiled bodies are involved in the processing of these specialized RNAs.

Although an earlier study had reported that the U2 snRNA genes do not associate with the coiled body in HeLa cells [Matera and Ward, 1993], there is now agreement on the fundamental observation that U2 genes associate with a substantial subset of coiled bodies [Frey and Matera, 1995; this study]. The HeLa monolayer cells used in this study were provided to Frey and Matera, who recently reported association with coiled bodies in both monolayer HeLa cells and their suspension HeLa cells, but with a higher frequency of association in the monolayer cells. In the HeLa suspension cells studied here we find 90% of cells have at least one U2 gene associated with the coiled body, supporting that the association is not related to whether cells are in monolayer or suspension. Frey and Matera [1995] included in their study several active genes on different chromosomes which showed no association with the coiled body, strengthening the conclusion that the association is specific and not simply a consequence of gene activity. Frey and Matera [1995] did not note a significant incidence of pairing of U2 genes or U1 and U2 genes at the coiled body and concluded that there was no significant association of multiple snRNA genes with the same coiled body. In contrast, we conclude that multiple snRNA genes associate at the coiled body with sufficient frequency to be highly significant and relevant to understanding coiled body function, as discussed above.

A curious aspect of these results is that 40% of U1 and U2 loci which appear to be paired with each other are not with a coiled body. It will be important to examine this type of association for other snRNA genes and to compare it to non-snRNA gene associations in order to address its significance. However, we believe that spatial association of unrelated genes from different chromosomes would be very rare; hence, the association of U1 and U2 genes observed is unlikely to be random. Hence, our results may reflect the transitory nature of the coiled body. The clear association of U2 genes with very small coiled bodies suggests the coiled body may begin its formation at snRNA loci and then possibly move away from that site. Rather than "bud-off" the nucleolus, as has been suggested [Raska et al., 1990], it is possible that the coiled body moves to the nucleolus. One can imagine that the coiled body could have multiple roles which might include some autoregulatory feedback mechanism to keep the production of the cell's most fundamental molecular machinery, such as spliceosomes, ribosomes, and nucleosomes, in balance with each other and with the demands for growth and proliferation. Whether the association of a gene with a coiled body has a role in either turning off or turning on snRNA transcription will require detailed analysis of RNA expression from individual alleles, currently in progress.

Whatever its function, the intriguing and apparently dynamic interactions of coiled bodies with specific genetic loci documented here will likely be explained only in terms of novel mechanisms which currently challenge the imagination and our knowledge of the nucleus. Deciphering these mechanisms may prove to have

fundamental significance not only for understanding nuclear compartmentalization but possibly for global regulation of cell function.

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