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Human genes encoding U3 snRNA associate with coiled bodies in interphase cells and are clustered on chromosome 17p11.2 in a complex inverted repeat structure

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ABSTRACT

Coiled bodies (CBs) are nuclear organelles whose morphological structure and molecular composition have been conserved from plants to animals. Furthermore, CBs are often found to co-localize with specific DNA loci in both mammalian somatic nuclei and amphibian oocytes. Much as rDNA sequences are called nucleolus organizers, we term these coiled body-associated sequences 'coiled body organizers' (CBORs). The only sequences that have been shown to be CBORs in human cells are the *U1*, *U2* and histone gene loci. We wanted to determine whether other snRNA genes might also act as CBORs. In this paper we show that human *U3* genes (the *RNU3* locus) preferentially associate with CBs in interphase cells. In addition, we have analyzed the genomic organization of the *RNU3* locus by constructing a BAC and P1 clone contig. We found that, unlike the *RNU1* and *RNU2* loci, *U3* genes are not tandemly repeated. Rather, *U3* genes are clustered on human chromosome 17p11.2, with evidence for large inverted duplications within the cluster. Thus all of the CBORs identified to date are composed of either tandemly repeated or tightly clustered genes. The evolutionary and cell biological consequences of this type of organization are discussed.

INTRODUCTION

U3 RNA is the most abundant of the small nucleolar RNAs (snoRNAs), with an estimated 2×10^5 copies/cell (1). U3 is essential for growth in yeast (2) and is known to be required for very early pre-rRNA processing events in both yeast and mammals (2–4). Along with U8 and U13, U3 RNA is one of only three known vertebrate snoRNAs to be transcribed by their own promoters (1); the vast majority of snoRNAs are processed from within the introns of upstream transcripts (5). U3 RNA is transcribed by RNA polymerase II, however, its promoter sequences lack TATA boxes and polyadenylation signals (reviewed in 6). Instead, initiation and termination of snRNA transcription occur by mechanisms different from those of other polymerase II transcripts (7–9).

Human *U3* genes are part of a multigene family located on chromosome 17p11–p12 (the *RNU3* locus), with an estimated 5–10 copies/haploid genome (10). However, further characterization of the *RNU3* locus has been hampered by the relatively small insert size of λ clones (10) and the instability of large insert YAC clones (11). We became interested in the organization of *U3* genes as part of a search for genomic loci that preferentially co-localize with coiled bodies (CBs) in human interphase cells.

Coiled bodies are nuclear organelles, conserved from plants to animals that contain high concentrations of small nuclear ribonucleoproteins (snRNPs) involved in a variety of cellular processes, including pre-mRNA splicing, rRNA processing and histone mRNA 3′-end maturation (12–14). Due to their similar molecular composition, CBs are thought to be the somatic equivalent of the sphere organelles found in amphibian oocyte nuclei (12). Although CB function(s) remains obscure, recent studies have shown that histone, *U1* and *U2* genes co-localize with CBs in human cells (15,16). Of particular significance is the fact that sphere organelles are often found adjacent to the histone gene loci on amphibian lampbrush chromosomes (17,18). Thus the co-localization of CBs and sphere organelles with specific DNA sequences appears to have been evolutionarily conserved.

We wanted to determine whether other snRNA genes might also function as so-called coiled body organizer regions (CBORs). One common theme among these CBORs is that the various genes are multicopy and are either clustered (histone) or tandemly repeated (*U1* and *U2*). Thus we screened human genomic libraries and isolated P1 and BAC clones containing *U3* genes. Using the clones for fluorescence *in situ* hybridization (FISH) we found that, like the *U1* and *U2* genes mentioned above, *U3* genes frequently associate with CBs in interphase HeLa and HT-1080 cells.

Each of the clones map to the same cytogenetic location (17p11.2) and can be assembled into an ~200 kb contig. There are three different *U3* genes within this contig, as well as the upstream control region for a putative fourth gene. Furthermore, we found that the genes and flanking sequences within this cluster are

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highly conserved. Two of the genes are located within a 90 kb inverted duplication. Each half of this 90 kb element has the same (mirror image) restriction map and blotting and sequence analysis of coding regions and end clones reveals extensive sequence identity throughout the repeat, suggesting either a very recent duplication event or the existence of mechanisms for maintaining sequence homogeneity throughout the cluster.

MATERIALS AND METHODS

Library screening

P1 phage bearing a 266 bp PCR fragment from the region immediately upstream of the *U3* coding region were identified within a pooled human genomic library by Genome Systems Inc. (St Louis, MO). The primers were GM1 (5′-GCTGTCATTCAG-TATTATGCTAAT-3′) and GM2 (5′-AATGATCCCTGAAAGTA-TAGTCTT-3′) (19). Ten of the 25 top pools had positive PCR signals. From these 10 positives three P1 phage (clones 2981, 2982 and 2983) were identified in the lower pools. A pooled human BAC library was purchased from Research Genetics (Huntsville, AL) and screened with the RNU3 primer pair. The primers, called RNU3-5′ (5′-TGGGGTGCAGTGGGGTAAC-3′) and RNU3-3′ (5′-TTCTCGCTCCCTCTTTTGTCGC-3′), produce a 1.1 kb band corresponding to positions –570 to +461 with respect to the start site of *U3* transcription. Four clones were identified: 28C2, 53L14, 212M14 and 374C9. Clones 28C2 and 374C9 were found to be sister clones, with identical inserts, and are called C2/C9 throughout the text. Similarly, 53L14 and 212M14 were also sister clones and are called L14/M14.

Characterization of P1 and BAC clones

P1 and BAC clone DNAs were restricted and analyzed by pulsed field gel electrophoresis (PFGE) using both CHEF-mapper and FIGE-mapper gel systems (BioRad, Burlingame, CA). FIGE was used primarily for fragments in the 1–150 kb size range. DNAs were fractionated in 1% agarose in 0.5× TBE with pulse times ranging from 0.1 to 6.8 s. Following electrophoresis, gels were stained with ethidium bromide, photographed and transferred to nylon membranes by standard methods. Filters were hybridized using either Church's buffer overnight (20) or Rapid-hyb buffer (Amersham, Arlington, IL) for 2.5 h. Initial washing was performed at room temperature in 2× SSC, 0.1% SDS for 15 min, followed by two stringency washes at 0.1 or 0.2× SSC, 0.1% SDS at 60°C for 20 min.

U3 cDNA and promoter region PCR products were radiolabeled using standard techniques. RNA end fragment probes were radiolabeled using T7 or SP6 RNA polymerase (Promega, Madison, WI). Riboprobes were routinely preannealed with 0.2 mg/ml (final) human C_0t1 DNA (Gibco-BRL, Gaithersburg, MD) for 5 min at 65°C prior to hybridization in order to quench cross-hybridization of interspersed repeated sequences.

Subcloning and DNA sequencing

In order to analyze the individual *U3* genes from the P1 and BAC clones PCR fragments were generated with the GM1/RNU3-3′ primer pair (–242 to +461). All of the PCR-based subcloning was done using a thermostable polymerase mixture that included an editing activity (e.g. Pfu polymerase; Stratagene, La Jolla, CA). Fragments were subcloned using the TA cloning system (pCRII vector; Invitrogen). In addition, a 3.5 kb fragment containing the *U3b*2 gene was amplified from P1 2983 using a T7 vector primer in combination with the RNU3-3′ primer and then TA subcloned. A 3.0 kb *Bam*HI fragment containing the *U3b*1/*b*2 gene pair from 2983 as well as two *Not*I–*Kpn*I end fragments from P1 clones 2981 (3.3 kb) and 2982 (5.0 kb) were isolated and subcloned by conventional techniques [pBluescript SK(–); Stratagene, La Jolla, CA]. Sequencing was accomplished using a Taq-based cycle sequencing system incorporating fluorescent dideoxy terminators and run on an automated ABI 377 sequencer (Applied Biosystems, Foster City, CA).

In situ hybridization and immunocytochemistry

Metaphase spreads were prepared from human lymphocyte cultures by standard methanol:acetic acid fixation. P1 DNA was nick translated with biotin-dUTP and slides were hybridized to metaphase spreads and washed essentially as described (21). HeLa and HT-1080 (a human diploid fibrosarcoma) cells were grown in monolayers on chambered glass slides, pre-extracted with Triton X-100 and then fixed with paraformaldehyde as described (15). Slides were incubated with anti-p80 coilin antibodies, fixed, denatured and then hybridized with the biotinylated U3 probe as described (15). Slides were scored for co-localization of *U3* DNA and p80 coilin fluorescence using a dual bandpass filter set (Chroma Tech., Brattleboro, VT). For each slide the numbers of red and green signals were counted as well as the number whose signals were at least partially overlapping. Digital imaging microscopy was performed as described (15).

RESULTS

Isolation of P1 and BAC clones

In addition to the major spliceosomal snRNPs, CBs are thought to contain U3 snoRNP (22,23). We wanted to see if, like *U1* and *U2* genes, CBs associated with *U3* loci. Previous studies had shown that there are roughly 5–10 functional *U3* genes in the haploid human genome and that the sequences flanking the genes are highly conserved (10,24). Using a PCR fragment from the 5′-flank (essentially the upstream control region) as a probe for *in situ* hybridization, Mazan *et al.* (19) detected labeling only on human chromosome 17p11–p12. As part of our ongoing effort to identify genetic loci that interact with CBs, we isolated three P1 and four BAC clones containing *U3* genes. Sequencing of PCR products or subclones thereof showed that each of them contained at least one *U3* gene. The *U3* coding regions on P1 clones 2981 and 2982 were identical, while some of the subclones derived from P1 2983 (as well as the BAC clones) differed at several positions, including some in the coding region. A schematic of the observed coding region changes is shown in Figure 1. The regions flanking the *U3* genes (both the 5′- and 3′-ends) in each of the clones were also extremely similar and were a close match to the sequence in the database (data not shown; accession nos AF020531, AF020534 and AF020535).

Human *U3* genes associate with coiled bodies in interphase cells

Consistent with the idea that *U3* genes are clustered, we found that the clones all mapped to the same cytological location when used as FISH probes (Fig. 2A and B). FLpter measurements (25)

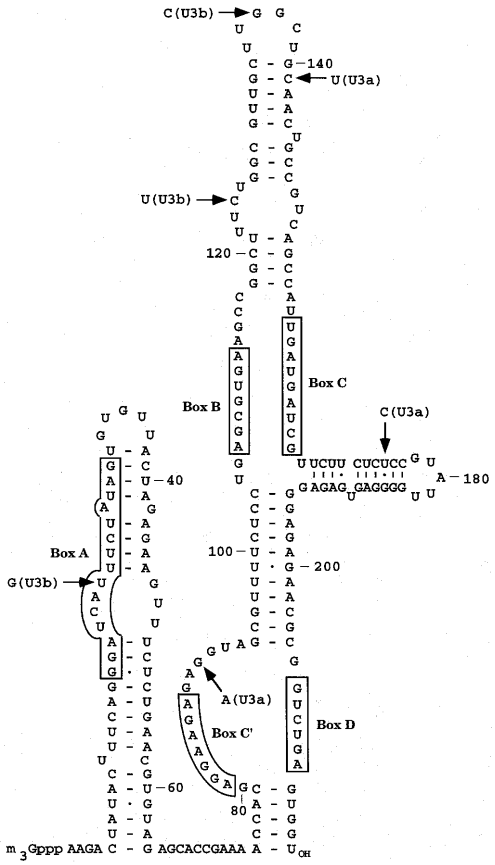

Figure 1. Sequence and secondary structure of human U3 RNA (adapted from 40). Site-specific changes in the *U3a* and *U3b* genes versus the RNA consensus sequence are marked with arrows. The conserved box A–D sequences are also shown.

on multiple metaphases averaged between 19.8 and 24.5% of the length of the chromosome and are completely consistent with previous mapping studies that placed the *RNU3* cluster at 17p11.2 (26). Once we had established that the clones contained *U3* coding sequences and that they hybridized to the proper cytogenetic location, we assayed the frequency with which the hybridization signals overlapped with CBs in both HeLa and HT-1080 cells (15). Interestingly, *RNU3* signals co-localized with at least one CB in ~54% of HeLa cells and 20% of HT-1080 cells (Fig. 2C). Previous work showed that histone, *U1* and *U2* gene signals co-localized with CBs in a similar fraction of cells, while those of control loci did not (15). The slightly lower rate of association of CBs with *U3* genes in HT-1080 cells is still significantly higher than that of the controls, especially given that the association rate of *U2* genes with CBs in HT-1080 cells is also reduced (~75% in HeLa versus 35% in HT-1080; M.R.Frey and A.G.Matera, unpublished observations). This lower rate likely results from the fact that HT-1080 cells are diploid and have an average of only 1–2 CBs/cell, whereas HeLa cells are notoriously aneuploid and have 4–6 CBs/cell (see 15 for a discussion).

Given the relatively small number of CBs per cell and the increasingly larger number of chromosomal loci with which these CBs co-localize (to date the *RNU1*, *RNU2*, *RNU3*, *HIST1* and *HIST2* loci), it seems likely that the observed association rates would require that at least some CBs might be shared by more than one locus. Previous attempts to address this question assayed genes on different chromosomes: the *U1* and *U2* loci on chromosomes 1p36 and 17q21 respectively. Frey and Matera (15) found that *U1* and *U2* signals co-localized in ~8% of the nuclei, while Smith *et al.* (16) detected overlapping *U1*/*U2*/CB signals ~12% of the time (20% paired *U1*/*U2* signals, 60% of which co-localized with CBs). These authors suggest that since the signals occupy a relatively tiny volume of the cell, a 12% rate of overlap is highly non-random and therefore significant. While we hesitate to make such a claim, it was clear that additional loci should be assayed. Hence, we measured the frequency of *U2* and *U3* gene signal overlap in interphase HeLa cells. Since these sequences are located on opposite ends of human chromosome 17 (~70 Mb in normal cells), it may be that these sequences co-localize more often. When assayed independently we found that 45% of all *U2* signals ($n = 342$) associated with CBs (15) and that 22% of all *U3* signals ($n = 582$) co-localized with CBs. Based on these individual probabilities we might expect that $0.45 \times 0.22 = 10\%$ of the *U2* and *U3* signals would overlap with CBs. We found that 33% of all the *U2*/*U3* signals were paired. Roughly 66% of the cells displayed at least one pair of signals. Clearly, additional multicolor analyses are needed before we can definitively answer this question, however, these data suggest that individual CBs may, indeed, associate with more than one DNA locus at a given time (Fig. 2D).

There are several properties that each of these putative CBORs (histone, *U1* and *U2* genes) have in common (15). They each encode non-polyadenylated polymerase II transcripts and have conserved 3′-terminal stem–loop structures. Furthermore, they do not contain introns and they are transcribed within large multicopy gene clusters in the human genome. We therefore wanted to learn more about the genomic organization of *U3* genes.

The *RNU3* gene cluster is complex and contains long inverted duplications

Our cytogenetic results (Fig. 2A) and those of Mazan *et al.* (19) suggested that *U3* genes are linked in the genome. However, Yuan *et al.* (10) isolated multiple independent λ phage clones and found that no two genes were present on the same clone. Hence, they inferred that *U3* genes must be at least 15–20 kb apart (10). Restriction mapping and Southern analysis of our P1 and BAC clones revealed that these large insert clones share extensive regions of homology (Fig. 3). PFGE showed that the three P1 clones were independent, while two of the BACs were sister clones to the other two (Fig. 3). The maps were constructed using a combination of *U3* coding region and promoter probes as well as various end fragment probes generated via T7 or SP6 transcription (see Materials and Methods). Examples of these blots are shown in Figure 4. In the first panel the three P1 clones were probed with a *U3* cDNA (Fig. 4A). When digested with *Mlu*I P1 clones 2981 and 2982 have only a single *U3*-positive band, whereas 2983 had three. All other enzymes tested showed only two bands for 2983. Analysis of the BAC clones showed that the L14/M14 sister clones had only two positive *Mlu*I fragments, despite otherwise similar restriction patterns (Fig. 4B). Visual inspection reveals, however, that one band is roughly twice as dark as the other one (e.g. Fig. 4B, lane 5). This suggested the existence of a nearly perfect inverted repeat. Fortuitously, one

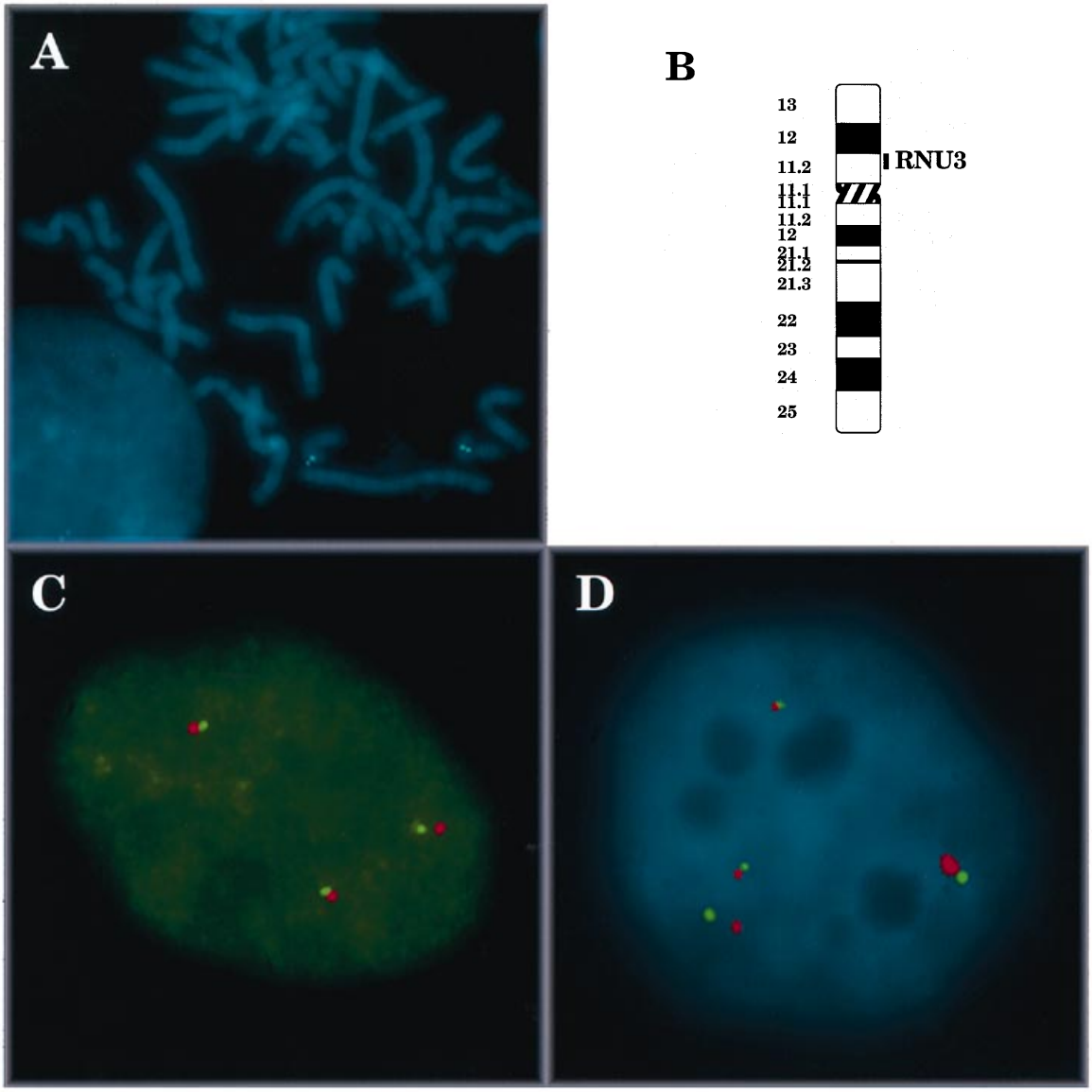

Figure 2. Cytogenetic mapping of *RNU3* clones and interphase association of the locus with CBs. (**A**) A human metaphase spread probed with P1 clone 2983. Signals (in green) were detected exclusively on chromosome 17p11.2 with all P1 and BAC clones. The DNA counterstain (DAPI) is shown in blue. (**B**) An ideogram of chromosome 17. The bar at right shows location of the FISH signals for the *RNU3* locus. (**C**) An interphase HeLa cell with two *U3* signals (green dots) overlapping with those of CBs (red). Approximately 54% of HeLa cells had ≥1 *CB*/*U3* gene co-localization (see text). More than 100 cells were scored for each association rate measurement. (**D**) An example of a cell with three sets of paired *U2* (green) and *U3* (red) signals and one upaired set. Despite being separated by ~70 megabases of linear DNA, *U3* genes exhibit a high rate of association with *U2* genes. Roughly 66% of the cells had at least one *U2*/*U3* signal overlap.

side of the inverted repeat was truncated within clone 2983, generating the third band (Fig. 3).

The extent of the inverted duplication is best illustrated by BAC clones L14/M14. A perfect mirror image of restriction sites projects nearly 45 kb in each direction from the two adjacent *Mlu*I sites near the inverted *U3* genes (Fig. 3). The duplicated region must end somewhere distal to the sp6/82 probe region, otherwise there would have to be an additional *U3* gene immediately distal to that region in the BAC clones (Figs 3 and 4C). In Figure 4C the same blot from Figure 4B was reprobed with the SP6 transcript from clone 2982, revealing the presence of two bands that co-migrate with most of the enzymes we tested (e.g. Fig. 4C, lanes 7 and 8), but not with *Mlu*I (Fig. 4C, lane 5). Thus the sp6/82 homology region is duplicated in L14/M14 and is very close to the end of the inverted repeat.

Sequence analysis reveals extensive similarity throughout the cluster

We next analyzed the regions around the *U3* genes at higher resolution using a combination of conventional gel electrophoresis, restriction mapping, subcloning of end fragments and DNA sequence analysis. The results are summarized in Figure 5. For clarity of discussion the solitary *U3* gene in the contig has

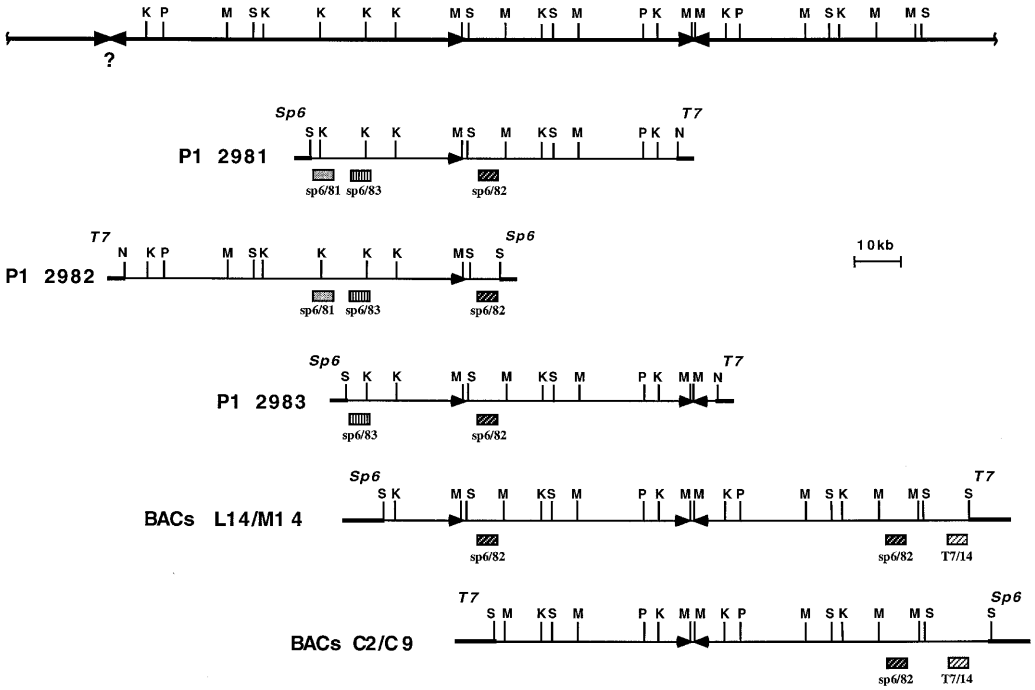

Figure 3. Schematic of the BAC and P1 contig for the *RNU3* locus. The top line is the contig map, the arrowheads mark the positions of the *U3* genes. Individual clones are marked at the left. The SP6 and T7 promoters are shown above the appropriate vector ends (thicker lines). Various *U3* promoter and coding region fragments (arrowheads), as well as T7 and SP6 end probes (hatched boxes) were used to construct the map. The question mark notes the probable location of an inverted *U3* gene pair, since sequence analysis of the end fragment from 2982 showed homology to the *U3* 5′-flanking region (see text and Fig. 6). Enzymes: K, *Kpn*I; P, *Pac*I; M, *Mlu*I; S, *Sal*I; N, *Not*I.

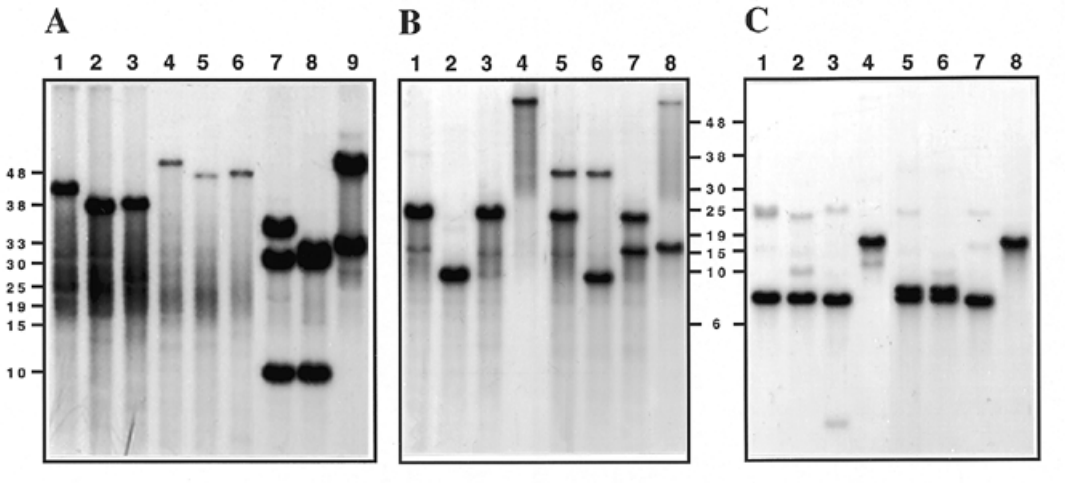

Figure 4. Southern blot analysis of P1 and BAC clones. (**A**) P1 clones 2981 (lanes 1–3), 2982 (lanes 4–6) and 2983 (lanes 7–9) were digested with *Mlu*I (lanes 1, 4 and 7), *Mlu*I/*Sal*I (lanes 2, 5 and 8) or *Sal*I (lanes 3, 6 and 9). The blot was hybridized with a *U3* cDNA probe. Clone 2983 clearly has three bands in lane 7, while the other clones have only one band. 2982 DNA was under-loaded relative to the others. (**B**) BAC DNAs C9 (lanes 1–4) and M14 (lanes 5–8) probed with a *U3* coding region probe. Clones were digested with *Mlu*I (lanes 1 and 5), *Mlu*I/*Pac*I (lanes 2 and 6), *Mlu*I/*Sal*I (lanes 3 and 7) and *Sal*I (lanes 4 and 8). Note that there are two bands in lanes 5–8. In each case one band is roughly twice the intensity of the other, due to inverted duplication of the *U3b* genes (see Fig. 5). (**C**) The blot from (B) was stripped and reprobed with an SP6 end fragment RNA probe from clone 2982 (marked sp6/82 in Fig. 3). Note that there are two regions of sp6/82 homology within BAC clone M14, but that only certain enzyme combinations reveal their presence (e.g. lanes 5 and 6). The SP6 RNA probe was preannealed with human C_0t1 DNA prior to hybridization to quench hybridization of repetitive DNAs, but some cross-hybridization (lighter bands) is still evident.

been designated *U3a* and the two inverted *U3* genes were named *U3b*1 and *U3b*2 (arrowheads, Fig. 5). Note that the restriction patterns of the flanking regions of the *U3a* and *U3b*1/*b*2 genes is conserved for over 2.5 kb upstream; downstream similarity extends for at least 700 bp (Fig. 5). Subcloning of a 3.5 kb end fragment from 2983, containing only *U3b*2, was accomplished using a T7 vector primer and a 3′-RNU3 primer. Analysis of this clone proved that the *U3b*2 gene is oriented as illustrated in Figure

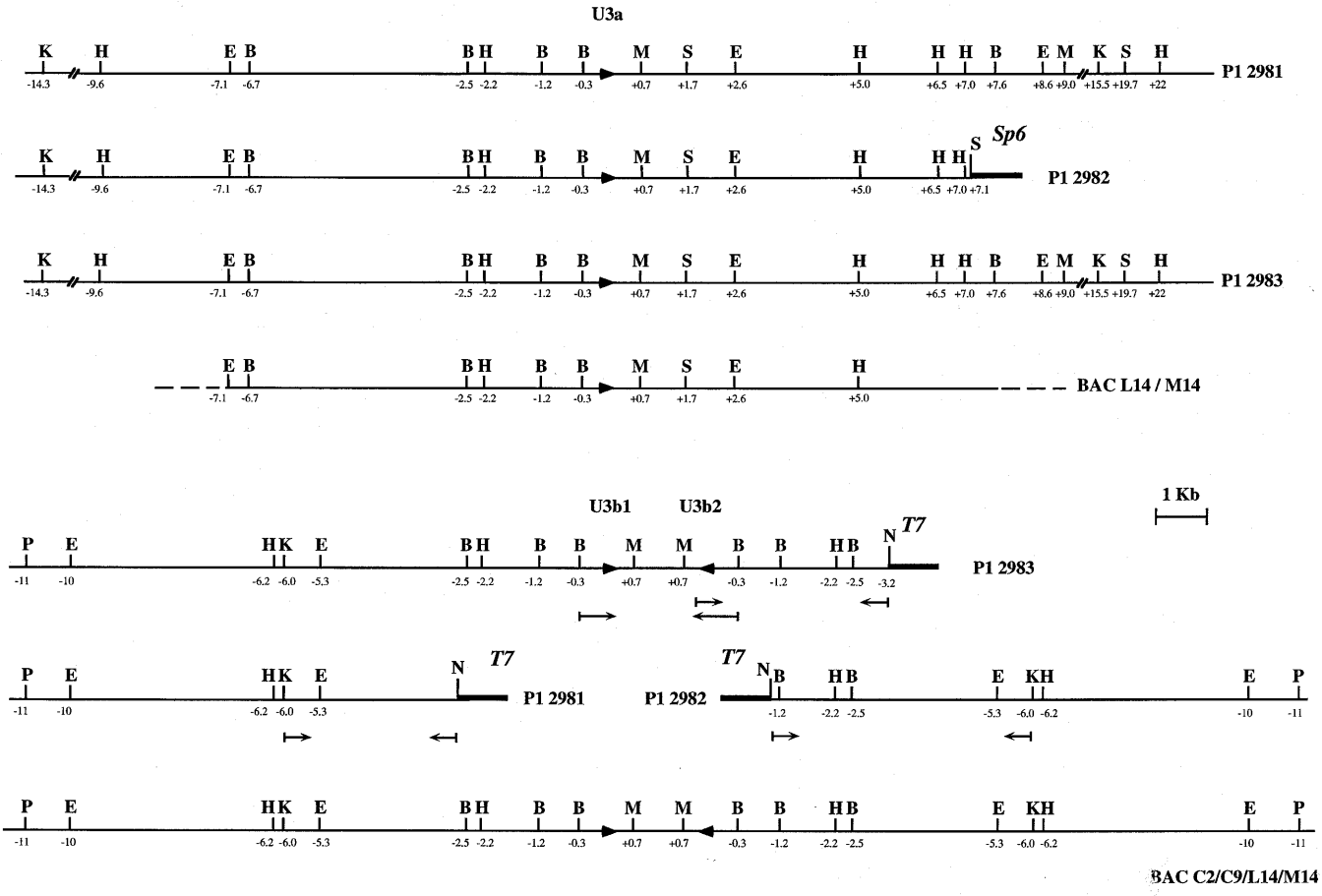

Figure 5. High resolution map of the *U3* flanking regions within the individual clones. The top four lines are alignments of the restriction patterns in and around the *U3a* gene. The bottom four lines show restriction maps for the *U3b* gene pairs. The numbers below the lines show the distance from the transcription start sites. Arrowheads mark the *U3* genes (all of which have been sequenced using PCR primers flanking the genes; see Materials and Methods), while arrows below the lines show additional regions that have been subcloned and sequenced. Thicker lines represent vector sequences; in the lower portion note that the left end of 2982 is juxtaposed to the right end of 2981 to save space and to illustrate their relative orientations only. Enzymes: K, *Kpn*I; H, *Hin*dIII; E, *Eco*RI; B, *Bam*HI; M, *Mlu*I; S, *Sal*I; N, *Not*I; P, *Pac*I.

3 and that its sequence is different from that of *U3a*, which we obtained by sequencing multiple PCR-derived clones from 2981 and 2982 (Fig. 1).

We also subcloned the *Kpn*I/*Not*I end fragments from clones 2981 and 2982. Sequencing from the *Kpn*I sites in both clones (which we know from PFGE must be >100 kb apart) showed that the sequences were nearly identical (data not shown; compare accession nos AF020532 and AF020530). Likewise, alignment of sequences near the *Not*I sites of 2981 and 2983 (see arrows, Fig. 5) also showed extensive similarity (compare accession nos AF020533 and AF020536). Thus the inverted duplication extends nearly 45 kb in each direction from the midpoint of the *U3b* gene pair and the sequence complementarity within each half suggests that this event occurred relatively recently in evolution.

An additional point of interest was the fact that the sequence from the *Not*I site of 2982 showed striking similarity to the 5′-flanking region of the *U3* gene in the database (Fig. 6). Thus clone 2982 was truncated ~1.2 kb away from a putative fourth *U3* gene in our contig. Furthermore, the orientation and sequence context of this region suggests the existence of another *U3b*-like gene pair. In support of this hypothesis, we found that restriction digestion of genomic DNA with *Pac*I showed that the 22 kb band on which the mirrored *U3b*1/*b*2 genes reside is two to four times as bright as the other *Pac*I fragments (Fig. 7). Furthermore, assembly of our nearly 200 kb contig predicts the existence of a 106 kb *Pac*I band that is not present on any of the individual clones. A band of that length is clearly detected by Southern analysis of a *Pac*I genomic digest (Fig. 7). Similarly, digestion of YACs from this region as well as genomic DNA with *Kpn*I produced the predicted 31 and 14 kb bands, in addition to others (see Fig. 5; data not shown). However, these restriction fragments are contained within individual P1 and BAC clones and thus do not help to confirm the contig map. Comparison of our contig with genomic DNA using other rare cutting enzymes (e.g. *Sfi*I and *Mlu*I) was confounded by the probable methylation of CG dinucleotides in the genomic DNA. *Pac*I is unique among 8-cutter restriction enzymes in that its recognition site contains only AT base pairs.

In summary, our data demonstrate that, like *U1* and *U2* genes, *U3* genes preferentially associate with CBs in interphase human cells. Along with the two histone loci on human chromosomes 1 and 6, the *RNU3* locus is the fifth such region to be identified as a CBOR.

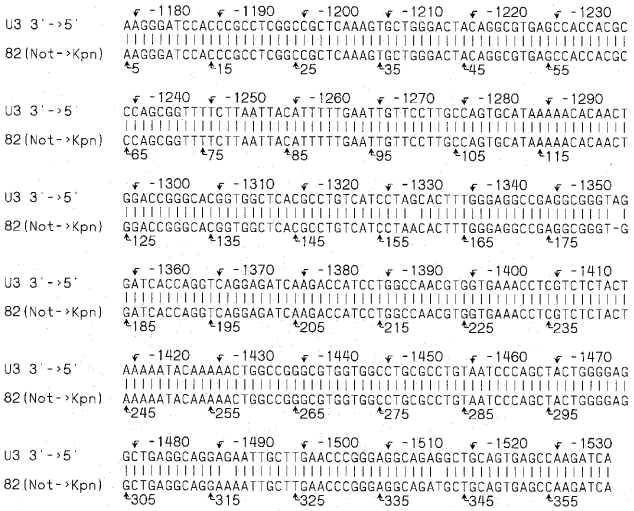

Figure 6. Sequence alignment of the end clone from 2982 (see Fig. 3; accession no. AF020529) reveals homology to the upstream control region of a *U3* gene (24) and suggests the existence of another *U3b* gene pair. Numbering is from the *U3* transcription start site (top strand) and from the vector cloning site in 2982 (bottom).

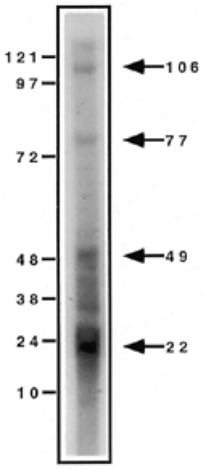

Figure 7. Genomic Southern analysis. High molecular weight genomic DNA was isolated according to established protocols, restricted with *Pac*I and subjected to FIGE. The blot was hybridized with a PCR fragment spanning positions –569 to +462 of the *U3* gene. Molecular weight markers are at left; four bands were detected and are marked with arrows at right. The 'band' at the top of the gel (≥150 kb) is due to a relatively large amount of DNA that is not cut by *Pac*I and was easily distinguishable on the ethidium stained gel (data not shown). Note that the 22 kb band is more intense than the others.

DISCUSSION

The *RNU3* locus

The high degree of sequence similarity within the inverted *U3b* repeats is remarkable. Not only is the restriction pattern identical over a stretch of nearly 45 kb in each direction, but sequence analysis of selected regions within the repeat showed >98.5% similarity at sites at least 6 kb upstream of the *U3* genes. Furthermore, pulsed field and conventional Southern analyses revealed that the repeat structure within the *RNU3* locus is complex. In addition to the solitary *U3a* gene and the inverted *U3b*1/*b*2 gene pair there is very likely an additional *U3b*-like gene pair at the 5′-end of our contig (see Figs 2, 6 and 7). The locations of the 49 and 77 kb bands are unknown, but likely to be nearby, since Mazan *et al.* (19) did not detect *U3* labeling at additional chromosomal sites.

Our high resolution clone maps (Fig. 5) agree well with those of Reddy and co-workers (10,24). These authors identified four different phage clones that look like *U3a* (HU3-1–HU3-4) and one clone whose map corresponds to half of a *U3b* inverted repeat (HU3-178). The authors comment on the fact that the gene in HU3-178 was truncated by the cloning vector and may be the result of an artefact (10). We suggest that copies of the inverted repeat were extremely under-represented in the older style λ library and thus only truncated versions of the *U3b* gene pair were obtained (10). Furthermore, our higher resolution maps agree well with previous genomic Southern analyses of the *RNU3* locus (10,26).

It was particularly surprising to find differences among the coding sequences of the *U3* genes. The sequences of the inverted *U3b* genes were identical, but differed at three positions from those of the published *U3* gene and at six sites with respect to the *U3a* gene (Fig. 1). Similarly, the *U3a* gene differed at three other sites from the published U3 RNA sequence. None of the changes interfered significantly with base pairing within stems of the U3 secondary structure (Fig. 1), however, one of the mutations (U23G in *U3b*) was in the conserved box A sequence. Although these differences may be attributed to sequencing artefacts, multiple independent subclones amplified from different genomic clones (e.g. 2981 and 2982) had the same sequence. Interestingly, Yuan and Reddy (10) noted that the rat Novikoff hepatoma cell line had three different length isoforms of U3 RNA, whereas HeLa cell U3 migrated as a single band. The point mutations shown in Figure 1 should not alter the length of the *U3* transcripts and thus are consistent with previous results. Regardless of whether these point mutations are real or not, these *U3* genes all have extensive 5′ and 3′ homologies and thus are each presumably active genes.

Concerted evolution of *RNU3*

The gene duplication(s) within the *RNU3* locus is of recent evolutionary origin. Not only are the inverted repeat sequences nearly perfect (>98.5% similar), but the *U3a* and *U3b* genes share significant upstream (>2.5 kb) and downstream (>0.7 kb) similarities. Additionally, the mouse *U3* gene cluster is located on chromosome 11, in a region that is known to be syntenic with human chromosome 17p (19). Three of the four *U3* genes from the mouse *rnu3* locus are located on the same cosmid clone and all are oriented in the same direction (27). Thus the *U3* genes appear to evolve in concert (i.e. different *U3* genes are more similar within a species than they are between species).

However, gene conversion events within the *RNU3* locus are apparently not as frequent as those within the *RNU2* locus on chromosome 17q21 (28–30), since *U2* genes within that array are essentially identical. Perhaps this greater variability among *U3* sequences is due to their clustered (as opposed to tandem) repeat organization. Indeed, concerted evolution may well be a consequence of tandem repetition (29,31).

Coiled body organizers

Early studies on amphibian oocyte nuclei revealed many morphologically distinct structures, including nucleoli, lampbrush chromosomes and 'spheres'. The sphere organelles were first identified structurally, as knobs attached to the lampbrush chromosomes of the newt (32, reviewed in 33). Of particular interest is the fact that some of the spheres within the amphibian oocyte are attached to the so-called 'sphere organizer' (SOR) regions of the lampbrush chromosomes. These SORs are located at or near the three different histone gene clusters (17,18) on the chromosomes of both frogs and newts. Spheres and CBs each contain high concentrations of ribonucleoproteins and other RNA processing machineries (reviewed in 13,34). One of the most important links between CBs and spheres is that SPH-1, an integral protein component of spheres, shows significant structural homology to p80 coilin (35). Furthermore, Gall and co-workers (36) demonstrated that the human p80 coilin protein is targeted to spheres when human mRNA was injected into amphibian oocytes. Thus spheres and CBs are homologous organelles that are likely to have similar functions, but to date no functions have been demonstrated (12).

We have shown that the *RNU3* locus preferentially associates with CBs in interphase human cells. All of the human CBOR loci identified thus far (*U1*, *U2*, *U3* and histone) are members of repetitive gene families. What factors nucleate CB formation? Is it an RNA- or DNA-based event? It is possible that, like nucleoli and rRNA genes, CBs may form as a *consequence* of transcription of these clustered genes. It is important to note that Sm and trimethylguanosine epitopes are present in CBs, hence, they are likely to contain mature, rather than nascent, snRNAs (13). Thus the analogy of NORs and CBORs is incomplete, since nucleoli form in direct response to transcription of rDNA (37), whereas at least some of the snRNAs within CBs are thought to travel to the cytoplasm for snRNP assembly prior to re-import into the nucleus (38). In the case of the histone gene–CB association it seems that CBs have the additional function of recruiting U7 snRNA, since the Sm site of U7 is required to specifically target this RNA to CBs following microinjection into *Xenopus* oocytes (39).

Moreover, p80 coilin may be involved in cellular processes affecting many other genes that are not recognized as CBORs. Perhaps the only reason we detect such high concentrations of p80 coilin near the CBORs is because these genes are clustered. Current efforts are underway in the laboratory to characterize additional single and multicopy snRNA gene loci and to ask whether they associate with CBs.

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