A Perinucleolar Compartment Contains Several RNA Polymerase III Transcripts as well as the Polypyrimidine Tract-binding Protein, hnRNP I

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Abstract. We have investigated the subcellular organization of the four human Y RNAs. These RNAs, which are transcribed by RNA polymerase III, are usually found complexed with the Ro autoantigen, a 60-kD protein. We designed 2′-OMe oligoribonucleotides that were complementary to accessible single-stranded regions of Y RNAs within Ro RNPs and used them in fluorescence in situ hybridization. Although all four Y RNAs were primarily cytoplasmic, oligonucleotides directed against three of the RNAs hybridized to discrete structures near the nucleolar rim. We have termed these structures "perinucleolar compartments" (PNCs). Double labeling experiments with appropriate antisera revealed that PNCs are distinct from coiled bodies and fibrillar centers. Co-hybridization with a genomic DNA clone spanning the human Y1 and Y3 genes showed that PNCs are not stably associated with the transcription site for these Y RNAs. Although 5S rDNA was often located near the nucleolar periphery, PNCs are not associated with 5S gene loci. Two additional pol III transcripts, the RNA components of RNase P and RNase MRP, did colocalize within PNCs. Most interestingly, the polypyrimidine tract-binding protein hnRNP I/PTB was also concentrated in this compartment. Possible roles for this novel nuclear subdomain in macromolecular assembly and/or nucleocytoplasmic shuttling of these five pol III transcripts, along with hnRNP I/PTB, are discussed.

THE nucleus, like the cytoplasm, is highly organized into discrete structural and functional domains. At the level of the light microscope, the most obvious structure in the interphase nucleus is the nucleolus, the site of rRNA transcription, processing and subunit assembly. With the use of antibodies and specific nucleic acid probes, it has become increasingly clear that many nucleoplasmic components, such as splicing factors and small ribonucleoprotein particles, are also nonrandomly distributed throughout the nucleus (Lamond and Carmo-Fonseca, 1993; Spector, 1993).

We have been studying a class of small ribonucleoprotein particles known as Ro RNPs. These RNPs were discovered because antibodies directed against these particles are present in many patients suffering from systemic lupus erythematosus and Sjogren's syndrome (Mattioli and Reichlin, 1974; Alspaugh and Tan, 1975; Lerner et al., 1981). Ro RNPs consist of several small cytoplasmic RNA molecules, each of which is complexed with a 60-kD protein (Wolin and Steitz, 1984). The RNA components, known as Y RNAs, are transcribed by RNA polymerase III and range in size from 69–112 nucleotides (Wolin and Steitz, 1983; O'Brien et al., 1993). In human cells, there are four distinct Y RNAs, called hY1, hY3, hY4, and hY5 (hY2 is a truncated version of hY1). Ro RNPs are present in mammalian cells at ~1% the level of ribosomes.

Although Ro RNPs have been described in a variety of vertebrate species (Hendrick et al., 1981; Reddy et al., 1983; Mamula et al., 1989; O'Brien et al., 1993), as well as in the nematode (Van Horn et al., 1995), their function has remained obscure. However, the 60-kD Ro protein is also found complexed with certain variant 5S rRNA precursors in *Xenopus* oocytes (O'Brien and Wolin, 1994). Because these 5S rRNAs are inefficiently processed to mature 5S rRNA and are eventually degraded, the 60-kD Ro protein has been proposed to play a role in a quality control or discard pathway for 5S rRNA biosynthesis (O'Brien and Wolin, 1994).

To investigate the subcellular distribution of Y RNAs in human cells, we have performed fluorescence in situ hybridization (FISH)[1] using antisense oligonucleotide probes. We find that while the majority of the human Y RNAs are

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1. *Abbreviations used in this paper*: FISH, fluorescence in situ hybridization; MRP, mitochondrial RNA processing; NGS, normal goat serum; PNC, perinucleolar compartments; pol I, polymerase I; 2′-OMe, 2′-O-methyl.

cytoplasmic, oligonucleotides complementary to three of the four human Y RNAs also hybridize to discrete structures at the edge of nucleoli. These structures, which we call "perinucleolar compartments" (PNCs) do not correspond to the site of transcription of Y RNAs or 5S rRNA and are distinct from coiled bodies. By performing double-labeling experiments, we show that PNCs are identical to the perinucleolar structures containing the polypyrimidine tract binding protein, hnRNP I/PTB, that were described by Ghetti et al. (1992). In addition to Y RNAs and hnRNP I/PTB, PNCs appear to contain two other RNA polymerase III transcripts, the RNA components of RNase P and RNase MRP.

Materials and Methods

Oligonucleotides, Antisera, and DNA Clones

All oligonucleotides directed against hY RNAs are listed in Table I. Oligonucleotides were synthesized at the Yale School of Medicine Protein and Nucleic Acid Chemistry Facility (New Haven, CT). 2'-OMe oligonucleotides were synthesized with either 2 biotin phosphoramidites (Bio-TEG; Glen Research, Sterling, VA) at either the 5' end [hY1(22-32), hY1(59-69), hY4(22-32), hY4(58-69), hY5(41-52), 4 biotin residues at the 5' end [hY3(51-65), hY3(57-70)], or 4 biotin residues at the 3' end [hY1(71-82), hY3(45-55), hY3(65-76)]. The digoxigenin-labeled 2'-OMe versions of hY1(71-82) and hY3(65-76) were prepared by incorporating 5' and 3' primary amine functionalities and conjugating with digoxigenin NHS esters (Boehringer-Mannheim Biochemicals, Indianapolis, IN) as previously described (Matera and Ward, 1992).

Other 2'-O-alkyl oligonucleotides used in this study consisted of anti-U3 (64-74), 5'-BBBBUUUCGGUGCUC-3' (Matera et al., 1994); anti-28S rRNA, 5'-AIAICCAAUCCUUAUdT-3' (Carmo-Fonseca et al., 1993; a gift of A. Lamond, EMBL, Heidelberg, Germany); anti-5S rRNA, 5'-BBBBCGGUAUUCCCAGGCG-3' (this probe differs by one nucleotide from that used by Carmo-Fonseca et al., 1993); anti-MRP RNA (Th7), 5'-AGCUGACGGAUGACGBBB-3' (Lee, B., A. G. Matera, D. C. Ward, and J. Craft, manuscript submitted for publication); anti-MRP RNA (Th12), 5'-GUAACU-AGAGGGAGCUBBB-3' (Lee et al., submitted); and anti-RNaseP RNA(H1-8a), 5'-CUGGCCGUGAGUCU-GUUCBBB-3' (Lee, B., A. G. Matera, D. C. Ward, and J. Craft, manuscript submitted for publication).

Monoclonal antibody 7G12 (a gift of G. Dreyfuss, University of Pennsylvania, Philadelphia, PA) was used for detection of hnRNP I/PTB. Anti-p80-coilin antiserum R508 and anti-fibrillarin monoclonal antibody 72B9 (gifts of E. Chan and E. Tan, Scripps Institute, La Jolla, CA) were used as markers for coiled bodies and nucleoli, respectively. Monoclonal anti-La serum A1 (Chan and Tan, 1987) was used for the La/PNC colocalization experiments. Previously characterized human autoantibodies against RNA polymerase I and the Ro 60-kD protein were provided by J. Craft (Yale University, New Haven, CT). Rabbit anti-sera against recombinant human Ro 60- and Ro 52-kD proteins were gifts of J. Keene (Duke University, Durham, NC).

A cosmid clone containing the human hY1 and hY3 genes was isolated from a genomic library (provided by S. Reeders, Brigham and Women's Hospital, Boston, MA) using the 3-kb Sal I/EcoRI single-copy subfragment of the genomic clone (Wolin and Steitz, 1983). This fragment contained the hY3 gene, but lacked the Alu sequence and hY1 gene. Digoxigenin-labeled oligonucleotide GM-009 (an Alu consensus oligomer; see Matera and Ward, 1992) was used to generate R-banded chromosomes for metaphase analysis. A plasmid clone containing one human 2.2-kb 5S rDNA repeat (a gift of R. Little, Albert Einstein, NY, NY) was used to mark the interphase location of human 5S genes (Little and Braaten, 1989; Sorensen et al., 1991).

Oligonucleotide-directed Cleavage and Selections

HeLa cells growing in suspension at 5×10^5 cells per ml (a gift of S. DeGregorio and J. Steitz) were washed twice in TBS (40 mM Tris-HCl, pH 7.5, 150 mM NaCl) and resuspended in TBS supplemented with 3 mM $MgCl_2$ at a concentration of 5×10^7 cells per ml. After sonication (2×30 s with a sonifier at setting 3; Branson Ultrasonics Corp., Danbury, CT), cell debris was removed by centrifugation for 30 min at 16,000 *g* in a refrigerated microcentrifuge.

For RNase H cleavage assays, the extract was divided into equal aliquots (~100 μl each) and the appropriate oligonucleotide and 2 U of RNase H (Boehringer-Mannheim Biochemicals) was added. After a 30-min incubation at 30°C, immunoprecipitation with anti-Ro patient sera was performed as previously described (Wolin and Steitz, 1984). RNAs extracted from the immunoprecipitates were precipitated with ethanol and labeled at the 3' end with [^{32}P]pCp (England et al., 1980). In addition, RNAs contained in supernatants from the immunoprecipitates were extracted, precipitated and labeled at the 3' end to determine if any abundant RNAs were targeted for digestion by the added oligonucleotide.

Selections with 2'-OMe oligonucleotides were performed as described by Barabino et al. (1989) with the following modifications. After preblocking and washing streptavidin-agarose beads (Pierce Chemical Co., Arlington Heights, IL) as described, the HeLa cell sonicate was preincubated with an aliquot of the beads (100 μl beads per ml of sonicate) for 30 min at 4°C. After a 30-s spin in a microfuge, the supernatant was removed, divided into equal aliquots (~100 μl each) and the appropriate 2'-OMe oligonucleotide was added. Following a 30-min incubation at 30°C, 20 μl of beads was added to each aliquot, the volume was raised to 500 μl with 250 mM wash buffer (Barabino et al., 1989) and the mixture was rotated for 30 min at 30°C. The beads were washed six times with 250 mM wash buffer, digested with proteinase K (2 mg/ml), eluted, and precipitated as described. Selected RNAs were visualized by labeling at the 3' end with [^{32}P]pCp.

Slide Preparation

HeLa monolayer cells were grown on chambered slides as described previously (Matera and Ward, 1993). Briefly, the cells were prepared by removing the medium from the slide chambers and rinsing in PBS-A (134.5 mM NaCl, 3.5 mM KCl, 10.5 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, pH 7.2). After fixation in 4% paraformaldehyde, 1× PBS-A for 10 min at room temperature, the slides were quenched in 0.1 M glycine in PBS-A for 5 min and then permeabilized in either 0.5% Triton X-100 in PBS-A for 5 min at room temp or in −20°C acetone for 10 min. Alternatively, slides were pre-extracted for 1 min in 0.25% Triton X-100 in PBS-A before fixation. Pre-extraction did not significantly alter the nuclear fluorescence patterns, although cytoplasmic labeling was drastically reduced. We found that the Triton pre-extraction step was essential for the simultaneous detection of RNA and DNA in order to reduce the nonspecific cytoplasmic background generated by nick translated genomic DNA probes (see below). After fixation, permeabilization and rinsing, slides were stored in PBS-A at 4°C for no longer than 1 wk before use.

In Situ Hybridization and Immunofluorescence

After equilibrating the slides in 4× SSC, 0.1% Tween-20 for 5–10 min at room temperature, cells were hybridized in 20 μl 4× SSC, 10% dextran sulfate, ~1 μg/μl competitor oligonucleotide (a random 22 mer), and 0.2 to 1.0 pmol/μl labeled probe oligomer. The probe was allowed to anneal under a 22 × 22 mm coverslip for 30 min at 37°C in a humidified chamber. Following hybridization, the slides were washed 3 × 5 min in 4× SSC, 0.1% Tween at 37°C, blocked in 250 μl of 4× SSC, 3% BSA, 0.1% Tween for 20 min, and then incubated with fluorochrome-conjugated avidin-DCS (Vector Labs, Burlingame, CA) or anti-digoxigenin F_{ab} fragments (Boehringer-Mannheim-Biochemicals) for 20 min. The unbound detector was washed off in 4× SSC, 0.1% Tween (3 × 5 min at 37°C) before counterstaining in DAPI (4,6-diamidino-2-phenylindole, 200 ng/ml in 4× SSC, 0.1% Tween; Boehringer-Mannheim Biochemicals) for 1 min. After destaining for 1–2 min, slides were mounted in a 2.3% wt/vol DABCO (1,4-diazobicyclo-2,2,2-octane; Sigma Chem. Co.), 0.1 M Tris, pH 8.0, 90% glycerol antifade solution.

Double labeling of RNA and protein was accomplished by hybridizing the oligonucleotide as described above and then equilibrating the slide in 10% normal goat serum (NGS), 1× PBS-A. The primary antibody was diluted in 200 μl of the same solution and incubated for 30 min before washing (2 × 10 min) in PBS-A. Fluorochrome-conjugated secondary antibodies were similarly incubated in 10% NGS, 1× PBS-A for an additional 30 min and washed in PBS-A before counterstaining and mounting as described above.

Experiments involving simultaneous detection of genomic DNA sequences and RNAs or proteins were performed on slides that were pre-extracted with detergent, using a method similar to that described by Carter et al. (1991). After equilibration in 4× SSC or PBS-A, pre-

extracted cells were either hybridized with an antisense oligonucleotide or incubated with the appropriate antiserum, respectively. The entire procedure was carried out as described above except that before counterstaining and mounting, the slides were fixed a second time in 4% paraformaldehyde to lock the reporter molecules into place and then quenched in 0.1 M glycine and rinsed. After dehydration through a graded ethanol series, the slides were air-dried, denatured in 70% formamide, 2× SSC for 2 min in an 80°C oven, dehydrated again in an ice-cold ethanol series, and hybridized with the appropriate digoxigenin-labeled genomic clone, essentially as described (Lichter et al., 1990).

Image Acquisition

Images were acquired using a Zeiss Axioplan (63×, 1.25 NA; 100×, 1.3 NA or 25×, 0.8 NA Plan Neofluar infinity corrected objectives) epifluorescence microscope equipped with a precision optical bellows system for continuous magnification adjustment and a cooled CCD camera (Photometrics, Tucson, AZ). The microscope was fitted with highly plane-parallel emission filters which limit image displacement to ±1 pixel when switching filter channels. Image acquisition was performed using CCD image capture (Yale University, New Haven, CT). Pseudocoloring and merging were accomplished using Gene Join (Yale University) or Registration (Biological Detection Systems) software running on an Apple Macintosh Quadra computer. Figs. 3–7 were composed either from the individual source or from merged images using Adobe Photoshop (Adobe Systems) and printed directly using a digital dye-sublimation printer (Kodak).

Results

Identification of Single-stranded Regions within Human Ro RNPs

To design probes for in situ hybridization, it was necessary to first identify regions of Y RNAs that are single-stranded within native Ro particles. We therefore synthesized deoxyoligonucleotides complementary to various regions of the four human Y RNAs. The regions of the Y RNAs targeted by these oligonucleotides are shown on potential secondary structure models for these RNAs (Fig. 1). These structures, which were drawn to maximize regions of homology between the human and *Xenopus* Y RNAs (O'Brien et al., 1993), have been largely supported by chemical modification and enzymatic probing (van Gelder et al., 1994) of naked RNA.

Each of these oligonucleotides was incubated with human HeLa cell extracts in the presence of *Escherichia coli* RNase H. We then performed immunoprecipitations with anti-Ro antibodies and visualized the Y RNAs by labeling them at the 3' end with ^{32}P-pCp (Fig. 2 *A*). This analysis revealed that a number of oligonucleotides were able to efficiently target individual Y RNAs for digestion with RNase H (Fig. 2 *A*). For example, oligonucleotides hY1(22-32), hY1(59-69), and hY1(71-86), which are complementary to three regions predicted to be single-stranded in hY1 RNA, specifically targeted hY1 RNA for digestion (Fig. 2 *A*, lanes *1–3*). Similarly, addition of either oligonucleotide hY4(22-32) or hY4(58-69) resulted in the complete cleavage of hY4 RNA. For each oligonucleotide, we also analyzed the total RNA profile from each sample to determine if any abundant RNAs were also targeted for digestion. (This can occur if the oligonucleotide is fortuitously complementary to another RNA.) For each oligonucleotide, the total RNA profile was unaffected (data not shown).

The data from these experiments are summarized in Table I. We found that the pyrimidine-rich loops that are predicted to form in each of the four human Y RNAs are all accessible to added oligonucleotides. In addition, an 11 nt sequence that is found in both hY1 and hY3 RNAs and predicted to be partly single-stranded in both RNAs was accessible to the complementary oligonucleotide (Table I, hY1[46-56] and hY3 [45-55]). As expected, hY3(1-14), an oligonucleotide synthesized against the predicted stem region of Y3 that contains the binding site for the 60-kD Ro protein was unable to target any of the Y RNAs for digestion. The primary sequence of this part of the stem is conserved among all four human Y RNAs; thus, the oligomer is also complementary to the first 12 nucleotides of hY1 and hY4 RNAs and can base pair with 10 nucleotides within hY5 RNA. Therefore, the stems of the other Y RNAs are similarly inaccessible to this oligonucleotide, at least in the RNP form.

We synthesized biotinylated 2'-O-methyl (2'-OMe) oligoribonucleotides complementary to most of the accessible regions and examined the ability of these oligonucleotides to specifically hybridize to their complementary RNAs. Stably hybridized oligonucleotides were detected by affinity selection with streptavidin agarose beads. The selected RNAs were examined by labeling them with ^{32}P-pCp. As shown in Fig. 2 *B*, many of the oligonucleotides

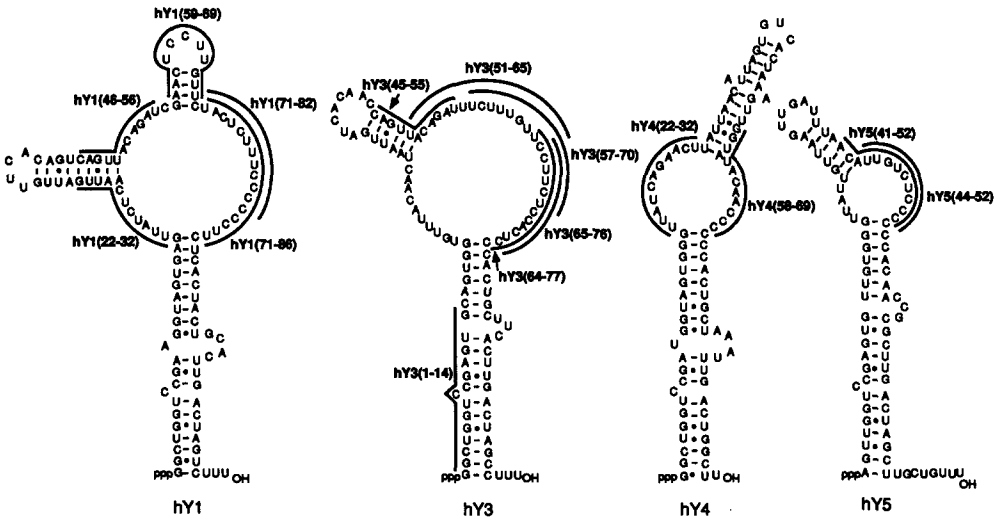

Figure 1. Potential secondary structures of Y RNAs. The structures shown were proposed by O'Brien et al. (1993). Oligonucleotides complementary to Y RNAs are indicated by bold lines.

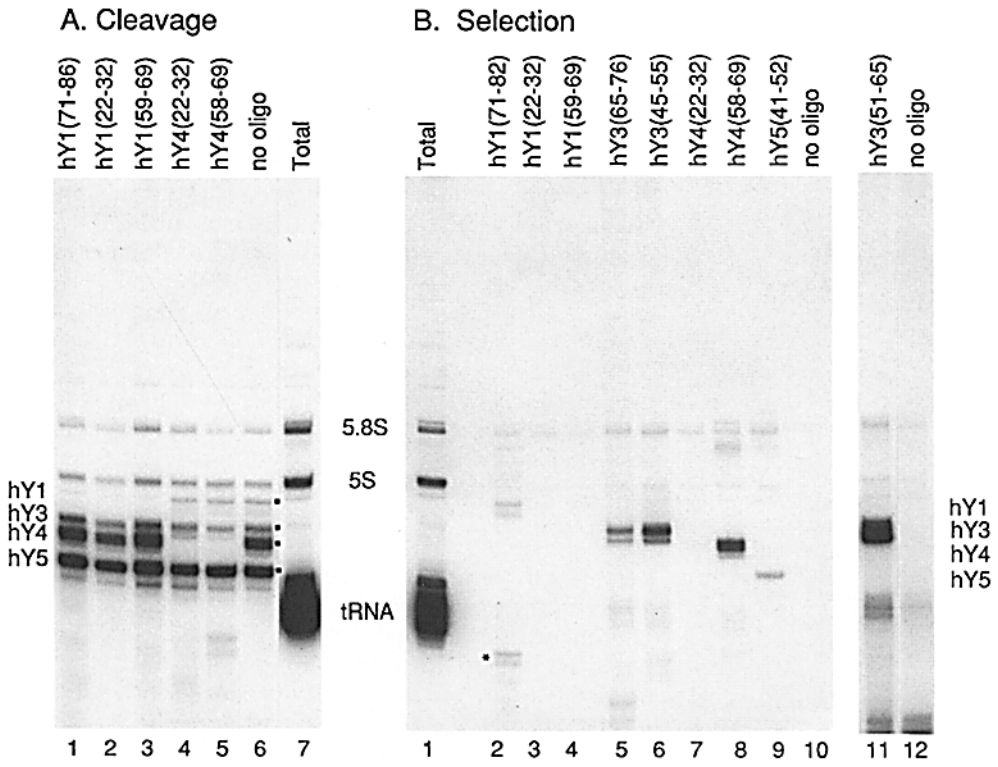

Figure 2. Identification of single-stranded regions of Y RNAs that are accessible to oligonucleotides within Ro RNPs. (*A*) Deoxyoligonucleotide-directed RNase H cleavage of human Ro RNPs. HeLa whole cell extracts were incubated with the indicated oligonucleotides and RNase H as described in Materials and Methods. Following incubation, the extracts were subjected to immunoprecipitation with human anti–Ro antibodies. RNAs contained in the anti-Ro immunoprecipitates were labeled with ^{32}P-pCp and fractionated in a denaturing gel (lanes *1–6*). The hY1, hY3, hY4, and hY5 RNAs are indicated by the dots adjacent to lane *6*. Lane *7*, total RNA was prepared by phenol extraction of a small portion of the lysate. The presence of 5S and 5.8S rRNA in all the immunoprecipitates is nonspecific, as these RNAs are also present in immunoprecipitates with nonimmune sera (not shown). (*B*). 2′-OMe oligoribonucleotide selection of Y RNAs. Equal aliquots of HeLa whole cell extracts were incubated with 50 pmol each of 2′-OMe oligonucleotides directed against either hY1 RNA (lanes *2–4*), hY3 RNA (lanes *5*, *6*, and *11*), hY4 RNA (lanes *7* and *8*) , hY5 RNA (lane *9*) or no oligonucleotide (lanes *10* and *12*). After incubation, oligonucleotide-bound RNPs were collected by addition of streptavidin agarose beads. Selected RNAs were examined by labeling at the 3′ end with ^{32}P. Total RNA is shown in lane *1*. The band indicated by the asterisk was identified as human mitochondrial tRNA^{ser}. Lanes *1–10* and lanes *11* and *12* represent separate experiments. The presence of 5.8S rRNA is many of the selections is nonspecific, as it appears in most lanes (regardless of the oligonucleotide used) and is also frequently found in selections containing no added oligonucleotides, such as that shown in lane 12. Note that hY1 (lane *2*) and hY3 (lanes *5* and *6*) migrate as doublets. The lower bands correspond to the hY1 degradation product, hY2, and the hY3 degradation product, hY3* (Wolin and Steitz, 1983).

specifically selected the appropriate Y RNA. However, one 2′-OMe oligonucleotide, hY1(71-82), selected an additional small RNA molecule that migrated slightly faster than the majority of the transfer RNA (Fig. 2 *B*, lane *2*). To determine the identity of this RNA, the band was excised from the gel and subjected to direct RNA sequencing. This analysis revealed that it was human mitochondrial tRNA^{ser} (de Bruijn et al., 1980), which contains seven nucleotides of complementarity to the hY1(71-82) oligonucleotide.

Certain regions that were targeted for RNase H–mediated digestion by oligodeoxyribonucleotides were inefficiently selected by the analogous 2′-O-methyloligoribonucleotides (Fig. 2 *B*, hY1[22-32], hY1[59-69], hY4[22-32]). This may be due to the fact that while four paired bases are sufficient to target degradation by RNase H (Donis-Keller, 1979), selection with 2′-OMe oligonucleotides may require greater complementarity. For example, the 5′ side of the large internal loop common to all Y RNAs was accessible in the RNase H experiments, but was not efficiently selected by the 2′-OMe versions of the same oligonucleotides (Fig. 2 *B*, lanes *4* and *7*, oligonucleotides hY1[22-32] and hY4[22-32]). However, the 3′ side of this loop was accessible to oligonucleotides in both assays. Similarly, the 2′-OMe oligonucleotide that is complementary to an RNase H accessible sequence present in both hY1 and hY3 RNAs (hY1[46-56] and hY3[45-55]) only selected hY3 RNA from cell extracts (Table I and Fig. 2 *B*, lane *6*). We therefore refer to this 2′-OMe oligonucleotide as hY3(44-55).

Human Y RNAs Appear To Be Present in the Cytoplasm and in a Perinucleolar Structure

We investigated the subcellular organization of hY1, hY3, hY4 and hY5 RNAs using the biotinylated antisense 2′-OMe oligoribonucleotides shown in Fig. 2 *B* and FISH. In these experiments, HeLa cell monolayers were fixed with paraformaldehyde, followed by permeabilization with acetone. To visualize the nuclei, cells were counterstained with DAPI. As expected, the distribution of all four of the human Ro RNAs was primarily cytoplasmic (Fig. 3, *A–L*). However, certain oligoribonucleotides directed against hY1, hY3, and hY5 RNA also stained novel structures near the periphery of nucleoli (Fig. 3, *A–H* and *K–L*). These structures appeared as one or two bright dots or tracks on the inner nucleolar border. We have termed these structures perinucleolar compartments (PNCs). Al-

Table I. Oligonucleotides Directed Against hY RNAs

Target	Sequence	Cleavage (DNA oligo)	Selection (2′-OMe oligo‖)	In situ (PNCs)
hY1(22-32)	AATTGAGATAA	+	−	ND
hY1(59-69)	AACAAGGAGTT	+	±	−*
hY1(71-82)	GGGGAAAGAGTA	ND	+	+
hY1(71-86)	AAGGGGGGAAAGAGTA	+	ND	ND
hY1(46-56)	ATCTGTAACTG	+	−	−
hY3(45-55)	ATCTGTAACTG	+	+	−
hY3(1-14)	CACTCGGACCAGCC	−	ND	ND
hY3(51-65)	GAACAAAGAAATCTG	ND	+	−
hY3(57-70)	AGAAGGAACAAAGA	ND	+	−
hY3(64-77)	GGAGTGGAGAAGGA	+	ND	ND
hY3(65-76)	GAGTGGAGAAGG	ND	+	+
hY4(22-32)	AGTTCTGATAA	+	±	−
hY4(58-69)	GGGTTGTATACC	+	+	−
hY5(41-52)	GGGAGACAATGT	ND	+	+
hY5(44-52)	GGGAGACAA	+	ND	ND

‖ 2′-OMe oligonucleotides also contained 2–4 biotin residues (see Materials and Methods).
+ Indicates >80% cleavage of the target RNA (for DNA oligonucleotides), >80% selection of the target RNA (for 2′-OMe oligonucleotides), or that >70% of HeLa cells contained at least one PNC (in situ hybridization).
± Indicates either weak (<10%) or variable selection efficiency.
* No staining of either PNCs or cytoplasm.

though not all nucleoli in a given cell contained PNCs (Fig. 3, compare *A* with *B* or *G* with *H*), the majority of HeLa cells (~70–90%) exhibited at least one PNC per cell.

Although oligomers directed against three of the four human Y RNAs hybridized both within the cytoplasm and the PNCs, there were subtle differences in their nuclear distribution patterns. When used at "high" concentrations (1.0 pmol/μl), the hY1(71-82) oligomer stained the entire nucleolus in addition to the PNCs (Fig. 3 *D*). At lower concentrations (0.2 pmol/μl), only the PNCs were visible (Fig. 3 *B*). The hY3(65-76) and hY5(41-52) oligonucleotide probes did not stain the entire nucleolus, even when used at 1.0 pmol/μl (Fig. 3, *F* and *H*). Additionally, the hY3(65-76) oligonucleotide revealed diffuse nucleoplasmic staining (Fig. 3 *H*) when used at 1.0 pmol/μl that was significantly reduced at the lower (0.2 pmol/μl) concentration (Fig. 3 *F*).

To determine if the PNCs recognized by oligonucleotides directed against hY5 RNA were identical to those recognized by oligonucleotides directed against hY1 and hY3 RNAs, we performed double-labeling experiments. Using digoxigenin-labeled hY1(71-82) and hY3(65-76) 2′-OMe oligomers (see Materials and Methods), we showed that the PNCs recognized by the different hY oligomers were indeed colocalized, although the overall labeling intensity was lower with the digoxigenin probes (Fig. 4, *A* and *B*).

Two oligonucleotides that efficiently selected hY3 from extracts did not hybridize to PNCs (Table I, hY3[51-65] and hY3[57-70]). However, these oligonucleotides did reveal both cytoplasmic and diffuse nucleoplasmic staining when used at 0.5 pmol/μl (data not shown). In addition, the hY3(44-55) oligonucleotide, which efficiently selects hY3 RNA from cell extracts (Fig. 2 *B*, lane *6*), gave only faint background staining in the FISH experiments (data not shown).

In addition to paraformaldehyde fixation and acetone permeabilization, we also tried a number of other extraction and permeabilization conditions (see Materials and Methods). We found that although the cytoplasmic signal was largely removed when the cells are extracted with 0.25% Triton X-100 before fixation, the PNCs remained undisturbed (Fig. 4, *C* and *E*). However, when cells are permeabilized with 0.5% Triton after the fixation step, the cytoplasmic staining was preserved (Fig. 4 *D*). Thus, while the cytosolic Y RNAs are soluble in Triton, the PNCs are not affected by detergent pre-extraction. Treatment of the cells with RNase prior to hybridization eliminated both the cytoplasmic and PNC staining, confirming that in both locations the oligonucleotides were hybridizing to RNA (data not shown).

Since Y RNAs are usually found complexed with the 60-kD Ro protein (Wolin and Steitz, 1984; Kelekar et al., 1994) we determined whether this protein was also present in PNCs. Indirect immunofluorescence experiments, using a human autoantibody that only recognizes the 60-kD protein on Western blots, revealed primarily cytoplasmic, but also weaker nucleoplasmic, staining (Fig. 4 *F*). Notably, the antibody did not stain PNCs (Fig. 4, compare *F* with *D*). We also obtained equivalent results (i.e., predominantly cytoplasmic staining without detectable PNCs) using an antibody raised against recombinant human 60-kD protein (Kelekar et al., 1994) (data not shown). Thus, it appears that a fraction of the Y RNAs, but not the 60-kD Ro protein, are located in this subnuclear compartment.

A 52-kD protein has also been proposed to be a component of Ro RNPs (Ben-Chetrit et al., 1988; Slobbe et al., 1992). However, using a variety of biochemical and immunological techniques, Kelekar et al. (1994) were unable to detect an association between the 52-kD protein and other components of Ro RNPs. To determine if the 52-kD protein might be associated with Y RNAs in PNCs, we per-

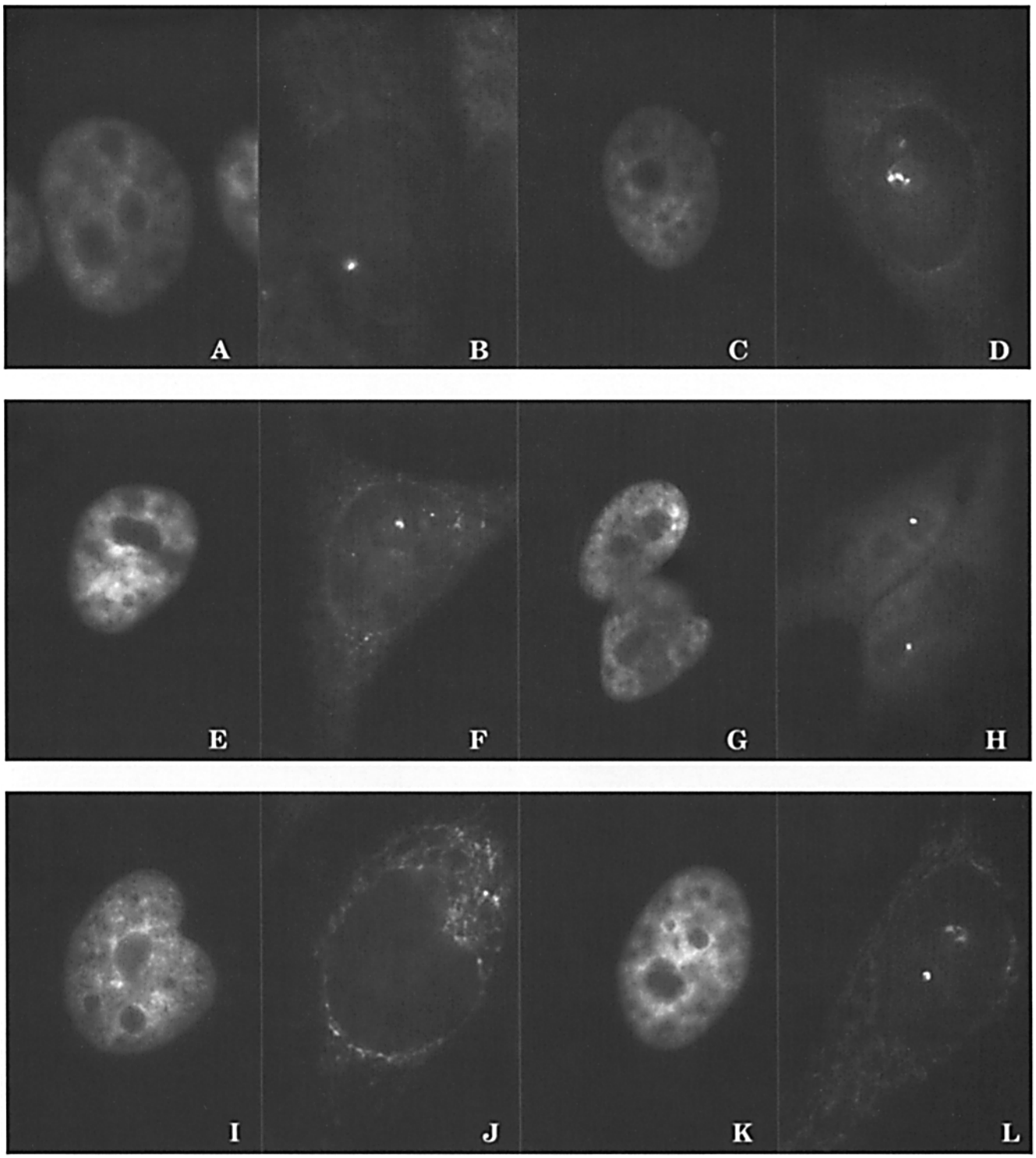

Figure 3. Fluorescence in situ hybridization with biotinylated antisense 2′-OMe oligonucleotides to HeLa monolayer cells. Cells were fixed in paraformaldehyde and permeabilized with acetone. Panels (*A, C, E, G, I,* and *K*) are the DAPI stained nuclei corresponding to the hybridization experiments shown in *B, D, F, H, J,* and *L*, respectively. *B* and *D* show hybridization of hY1(71-82) oligos at low (*B*, 0.2 pmol/μl) and high (*D*, 1.0 pmol/μl) probe concentrations. Fluorescence is detected throughout the cytoplasm and in PNCs, which are typically located on the inner periphery of the nucleolus. At higher probe concentration (*D*), a general low level of hybridization is detected in the rest of the nucleolus. Usually only one or two nucleoli per cell displayed PNCs. (*F* and *H*) Same as *B* and *D*, except with hY3(65-76) oligonucleotides. Note the lack of staining throughout the body of the nucleolus and the presence of nucleoplasmic hybridization at the higher probe concentration (*H*). *J* reveals that hY4(58-69), at 1.0 pmol/μl, is detected only in the cytoplasm. (*L*) The hY5(41-52) oligomer, also at 1.0 pmol/μl, is detected in the cytoplasm and PNCs, but not in the general nucleolar area.

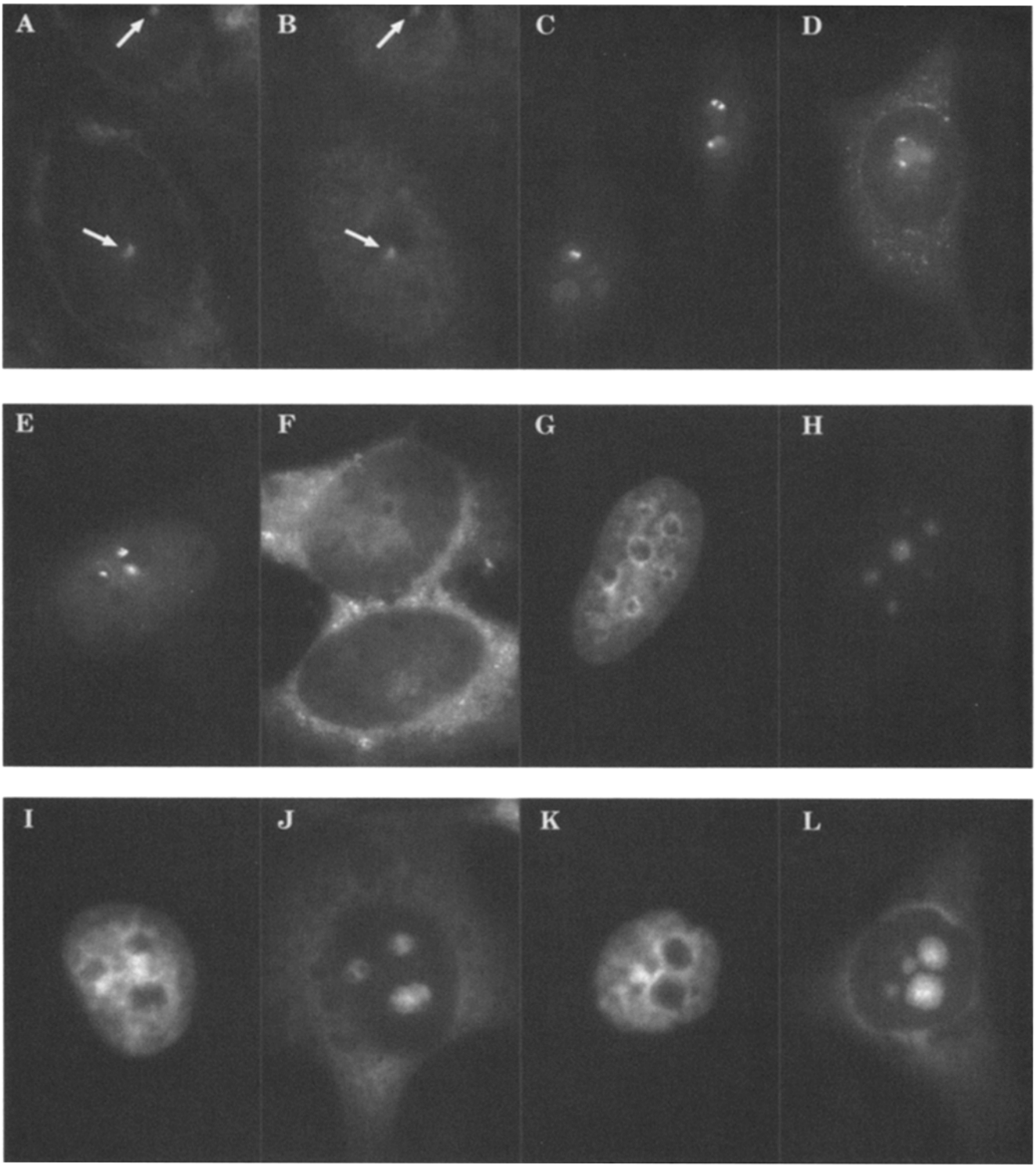

Figure 4. Experimental controls. The images in *A* and *B* reveal that the PNCs labeled by the biotinylated hY5(41-52) probe (*A*, *arrows*) are identical to those stained by the digoxigenin-labeled hY3(65-76) oligomer, (*B*, *arrows*). The cells in *C* and *D* are both hybridized with the same hY1(71-82) oligoprobe, but those in *C* were extracted with Triton X-100 before fixation and those in *D* were extracted after fixation. Note that although most of the cytoplasmic signal is removed by pre-extraction, the nucleolar staining remains relatively undisturbed. *E* shows that pre-extraction also does not disturb PNCs visualized with hY3(65-76). In *F* cells were stained with a human serum that recognizes only the 60-kD Ro protein on Western blots. Fluorescence was detected primarily in the cytoplasm, and not in the PNCs. (*G–L*) are control hybridizations. *G, I,* and *K* are the DAPI stained nuclei corresponding to the hybridization experiments *H* (anti-U3), *J* (anti-28S), and *L* (anti-5S), respectively. We sometimes observed local concentrations of 5S signal within nucleoli (*L*). Double-labeling experiments with anti-hnRNP I serum (data not shown, but see Fig. 7) demonstrated that these nucleolar regions enriched in 5S rRNA do not correspond to PNCs.

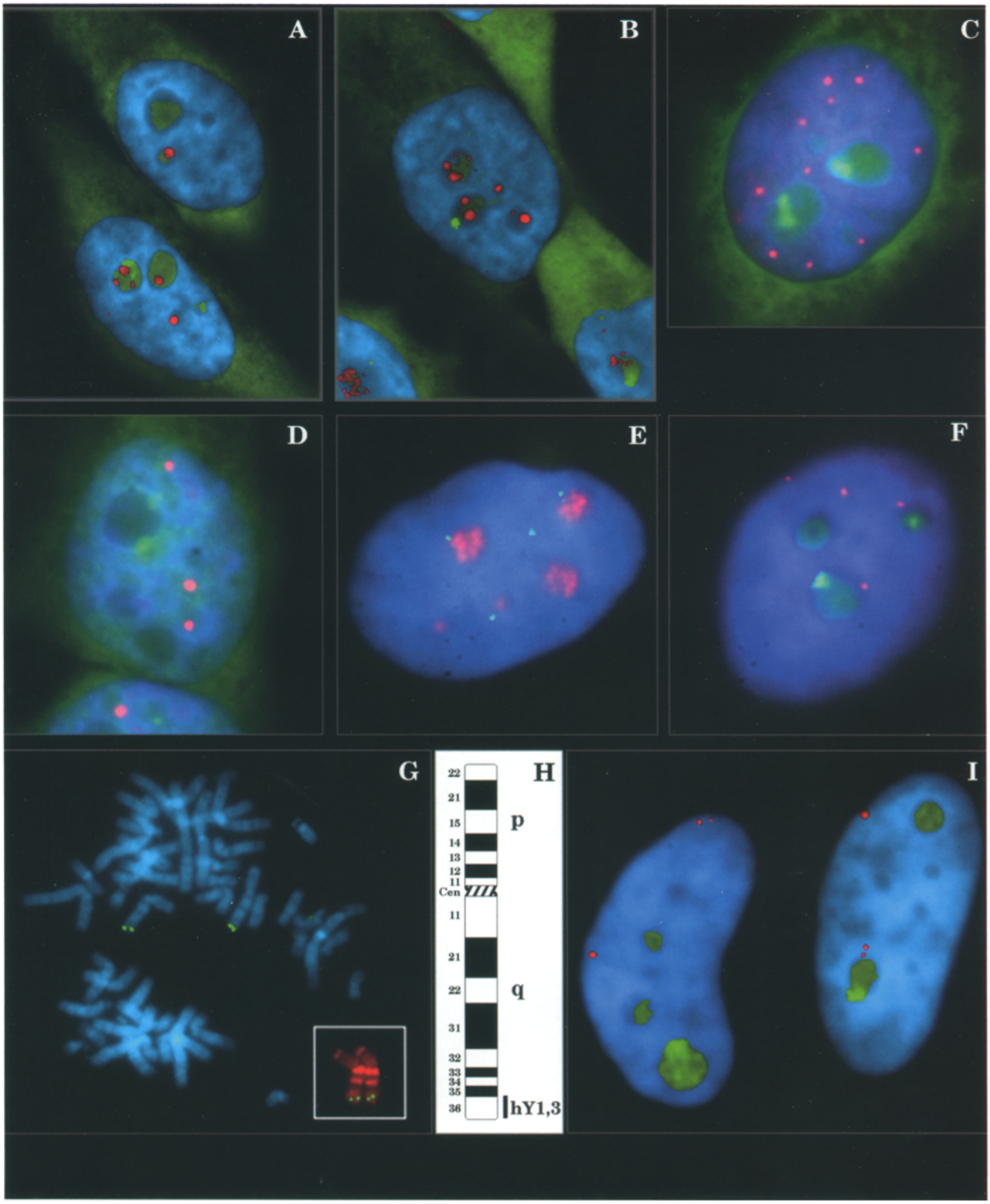

Figure 5. PNCs do not colocalize with other nuclear structures. Comparison of hY1(71-82) (*A*) and hY3(65-76) (*B*) hybridization patterns (shown in *green*) with immunofluorescence using anti-RNA polymerase I antibodies (*red*). The fibrillar centers (the large spheroid structures) are not colocalized with PNCs. *C* and *D* are the same as *A* and *B* except that anti-p80-coilin antibodies (*red*) were used. Coiled bodies were also found to be distinct from PNCs. (*E*) 5S rDNA loci (*green*) were often located near the nucleolar periphery. Nucleoli are marked using anti-fibrillarin mAbs (*red*). (*F*) Although 5S rDNA loci (shown in *red*) are often associated with the nucleolar periphery, they do not colocalize with the PNCs (*green*), visualized with the hY1(71-82) oligomer. (*G*) Metaphase chromosome analysis reveals that the genes for hY1 and hY3 RNA are located on chromosome 7 (signals shown in *green*). (*Inset*) On separate metaphase

formed immunofluorescence experiments using an antibody raised against recombinant human 52-kD protein (Kelekar et al., 1994). As was previously observed by Kelekar et al. (1994), we obtained predominantly nucleoplasmic fluorescence, and did not detect PNCs (data not shown).

PNCs Are Not Enriched in Nucleolar Components and Are Distinct from Coiled Bodies

Because PNCs are found at the edge of nucleoli, it was possible that they were enriched in nucleolar components. Control hybridizations with oligomers specific for the U3 small nucleolar RNA as well as for 5S and 28S ribosomal RNAs (Matera et al., 1994; Carmo-Fonseca et al., 1993) demonstrated that although these RNAs are present within nucleoli, they do not concentrate in PNCs (Fig. 4, *G–L*). RNA polymerase I (pol I) is concentrated within a region of the nucleolus known as the fibrillar center (see Matera et al., 1994 and references therein). As shown in Fig. 5 (*A–B*), double-labeling experiments using antibodies against pol I and hY oligomers revealed that PNCs do not correspond to these nucleolar subregions.

Many members of the U class of small nuclear RNPs are enriched in nuclear organelles known as coiled bodies (reviewed by Lamond and Carmo-Fonseca, 1993). As coiled bodies are frequently located near nucleoli, it was possible that the PNCs we detected using hY oligomers were actually coiled bodies. Double labeling experiments with antibodies against p80-coilin, a specific marker for coiled bodies (Andrade et al., 1991) and the hY oligomers showed that PNCs also do not colocalize with coiled bodies (Fig. 5, *C–D*).

PNCs Are Not Stably Associated with Sites of Either hY or 5S RNA Transcription

The genes encoding hY1 and hY3 are present as single copies in the human genome and are located within 3 kb of each other (Wolin and Steitz, 1983). In addition, Maraia et al. (1994) recently showed that the hY4 gene is located within 200 kb of these genes and that all four human hY RNA genes reside on chromosome 7. Thus, it was possible that the PNCs represented nascent transcripts from these tightly linked genes. Since the previously described 4-kb restriction fragment containing the hY1 and hY3 genes (Wolin and Steitz, 1983) was too short to be reliably detected in interphase cells, we used this fragment to isolate a cosmid clone containing these two genes. After mapping the clone on human metaphase chromosomes to 7q36, (Fig. 5, *G* and *H*), we performed a sequential hybridization experiment (see Materials and Methods). We first hybridized biotinylated hY1(71-82) and hY3(65-76) oligomers to detect PNCs, and then hybridized a digoxigenin labeled cosmid probe to detect the Y RNA genes. As shown in Fig. 5 *I*, PNCs are not stably associated with the site of hY1 and hY3 transcription (see also Table II).

*Table II. Hybridization Data**

Probes	% Associated‡	Average number of signals§
hY1 RNA/5S DNA	0	ND/3.7 ± 0.5
hY DNA/5S DNA	4	1.6 ± 0.6/3.6 ± 1.0
hY DNA/hY RNA	0	1.5 ± 0.8/ND
Nucleoli/5S DNA	53	ND/3.4 ± 1.0

* Slides scored using dual bandpass filter set and 100× objective.
‡ Fraction cells showing at least one association.
§ Values ±1 standard deviation, $n \geq 75$.

In *Xenopus* oocytes, the 60-kD Ro protein is found complexed with certain variant 5S rRNA molecules (O'Brien and Wolin, 1994). These 5S RNAs contain internal point mutations and are longer at the 3′ end. Although the Y RNAs have not been demonstrated to be present in the complex between the 60-kD Ro protein and defective 5S rRNA precursors, it was nonetheless possible that PNCs might correspond to the site of 5S RNA transcription. Using a probe for the 5S rDNA gene cluster on chromosome 1q42 (Little and Braaten, 1989; Sorensen et al., 1991), we performed double labeling experiments. Interestingly, 5S rDNA was often found to be located at the nucleolar periphery (Fig. 5, *E* and *F*), as was previously observed in the plant *Pisum sativum* (Highett et al., 1993). Using antifibrillarin as a nucleolar marker protein (the outline of the nucleolus in DAPI is much less distinct in denatured cells) we found that >50% of the signals (see Table II) were associated with the nucleolar border. However, in double-labeling experiments with hY oligomers (Figure 5, *F*), we failed to detect an association of 5S rDNA with PNCs (Table II).

Two Other pol III Transcripts Appear To Be Located in PNCs

Although the Y RNAs and 5S rRNA are all transcribed by RNA polymerase III, neither hY4 RNA nor 5S rRNA was found to concentrate in PNCs (Figs. 3, *I* and *J* and 4, *G* and *H*). In addition, hybridization with oligonucleotides specific for U6 RNA and 7SK RNA did not detect PNCs (Matera and Ward, 1993). We decided to examine some additional pol III RNAs to see if they were concentrated within PNCs. Although we designed several oligonucleotides against 7SL RNA, the RNA component of the signal recognition particle (Walter and Blobel, 1982), none of these oligonucleotides selected more than 1–2% of the 7SL RNA from cell extracts (unpublished data). Hybrid-

spreads, an Alu consensus oligonucleotide (*red*) was used to create an R-banded karyotype. The signals (*green*) reveal that the genes are located in band 7q36. (*H*) An ideogram of human chromosome 7 showing the range of the observed hybridization signals (*bar*). (*I*) The cosmid clone containing the hY1 and hY3 genes (*red*) was used in conjunction with the hY1(71-82) probe (*green*) to reveal that PNCs are not stably associated with the site of hY1 and hY3 transcription. See Table II for details of the hybridization scoring experiments. Because the oligonucleotides were used at 1.0 pmol/μl, the hY1 oligomer stains the entire nucleolus as well as PNCs in *A, C,* and *I.* In each of the panels, the cells were counterstained in DAPI, shown in *blue.* The cells in *E, F,* and *I* were pre-extracted with detergent in order to visualize the genomic DNA probes; cells in *A–D* were post-extracted. Images in *C–F* were merged using an algorithm that blends the colors of the three source images, while those in *A, B, G,* and *I* were merged using an algorithm that compares each pixel of the various source images and displays the color of the source that has the highest (normalized) intensity.

ization with two of these oligoribonucleotides revealed primarily cytoplasmic fluorescence, devoid of PNCs (data not shown). However, oligonucleotide probes complementary to two additional polymerase III transcripts did concentrate in PNCs. RNase mitochondrial RNA processing (MRP) is an enzyme that cleaves a primer RNA substrate involved in mitochondrial DNA replication (Clayton, 1994). The yeast homologue of the mammalian RNP is required for proper maturation of 5.8S rRNA (Schmitt and Clayton, 1993; Chu et al., 1994). The RNA components and at least one protein subunit of both the nuclear and cytoplasmic versions of RNase MRP are identical (Gold et al., 1989). This common protein (called the Th or To antigen) is also a part of RNase P, a related RNP enzyme that cleaves the 5′ ends of pre-tRNA precursors (Darr et al., 1992; Altman et al., 1993). Strikingly, when biotinylated 2′-OMe antisense probes specific for either MRP RNA (a.k.a. 7-2 RNA) or RNase P RNA (a.k.a. 8-2 RNA) are hybridized in situ, structures similar to PNCs are detected (Fig. 6, *A–D*; and Lee, B., A. G. Matera, D. C. Ward, and J. Craft, manuscript submitted for publication).

Double-labeling experiments with biotinylated MRP RNA probes and digoxigenin-labeled hY3 probes demonstrated that they labeled the same perinucleolar structures (not shown). However, double-labeling experiments using antibodies directed against the 40-kD Th protein did not detect this protein in PNCs (unpublished data and Lee et al., manuscript submitted for publication). Thus, while antisense oligonucleotides directed against MRP RNA, RNase P RNA and three of the four human Y RNAs decorated PNCs, we did not detect the known protein components of any of these RNPs in the perinucleolar structures.

As all of the oligonucleotides that stain the PNC are directed against polymerase III RNAs, we asked whether the La protein, which associates at least transiently with nascent pol III transcripts (Rinke and Steitz, 1982), is colocalized in PNCs. Using a monoclonal antibody (Chan and Tan, 1987), we found that anti-La staining was primarily nucleoplasmic on both pre- and post-extracted HeLa cells. While we detected a low level of nucleolar fluorescence in some cells, the pattern was uniform throughout the nucleolus and was not concentrated in PNCs (Fig. 6, compare *E* and *F*). These data, along with our failure to detect an association of Y RNA genes with PNCs, suggest that the RNAs located within PNCs represent neither nascent transcripts nor mature RNPs.

The Polypyrimidine Tract-binding Protein hnRNP I Colocalizes with PNCs

A protein that was initially characterized because it could be crosslinked to the polypyrimidine tract of intervening sequences in pre-mRNA (Garcia-Blanco et al., 1989; Gil et al., 1991; Patton et al., 1991) was later found to be identical to an isoform of hnRNP I (Ghetti et al., 1992). Although the precise function of hnRNP I/PTB remains unclear, immunofluorescence experiments using a monoclonal antibody revealed that in addition to a more general nucleoplasmic and cytoplasmic staining, the protein is concentrated in a discrete perinucleolar structure (Ghetti et al., 1992; Hellen et al., 1993). To test whether the hnRNP I clusters are identical to PNCs, we performed double-label-

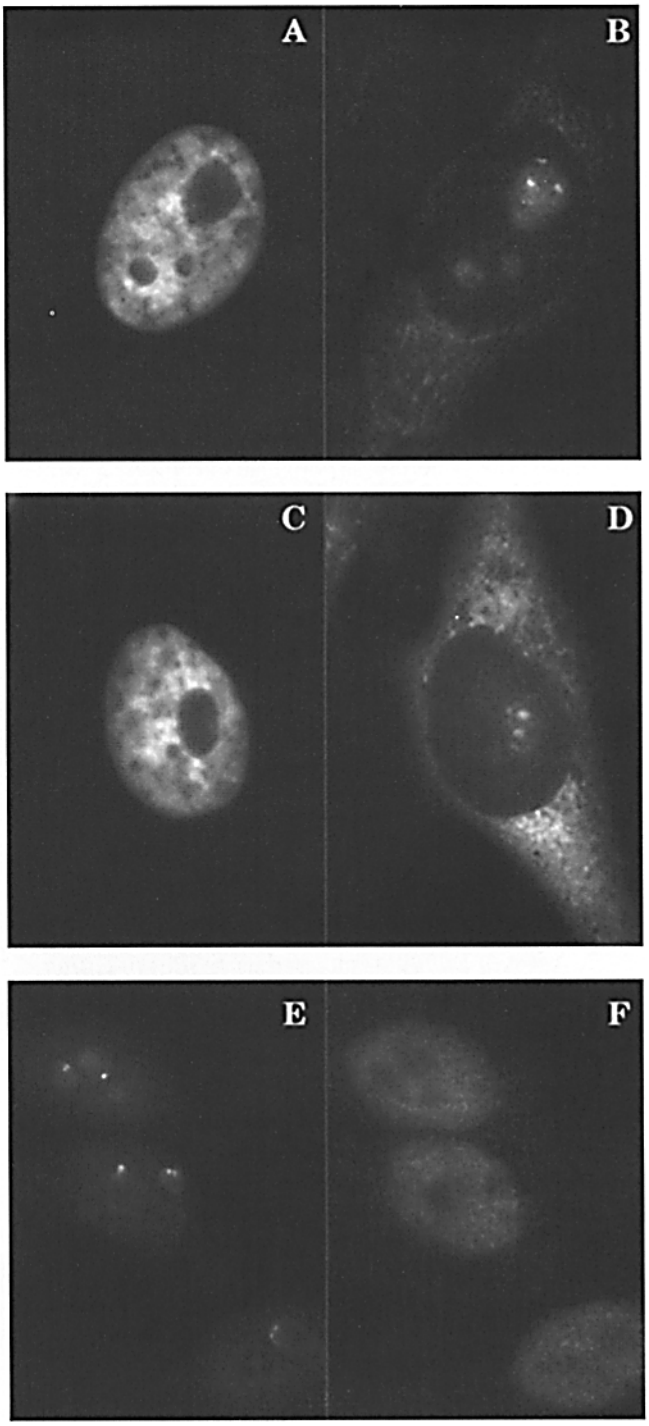

Figure 6. Two other pol III RNAs are concentrated within PNCs, but the La protein is not. (*A–D*) Post-extracted HeLa monolayer cells counterstained in DAPI (*A* and *C*) or hybridized with (*B*) anti-RNase MRP(Th7) or (*D*) anti-RNase P(H1-8a) oligoprobes. The RNase MRP oligonucleotide hybridizes mainly in the nucleolus and to a lesser extent in the cytoplasm, but is concentrated within PNCs. Conversely, the RNase P oligoprobe is predominantly cytoplasmic, with a lesser degree of nucleolar hybridization, but is also concentrated in PNCs. The cytoplasmic location of RNase P was somewhat unexpected, as tRNA maturation is thought to occur in the nucleus. However, a predominantly nuclear location for RNase P has never been demonstrated. In panels (*E* and *F*), pre-extracted cells were hybridized with hY1(71-82) (*E*) and stained with anti-La mAb A1 (*F*) . Note that anti-La is predominantly nucleoplasmic, with no apparent clustering in PNCs.

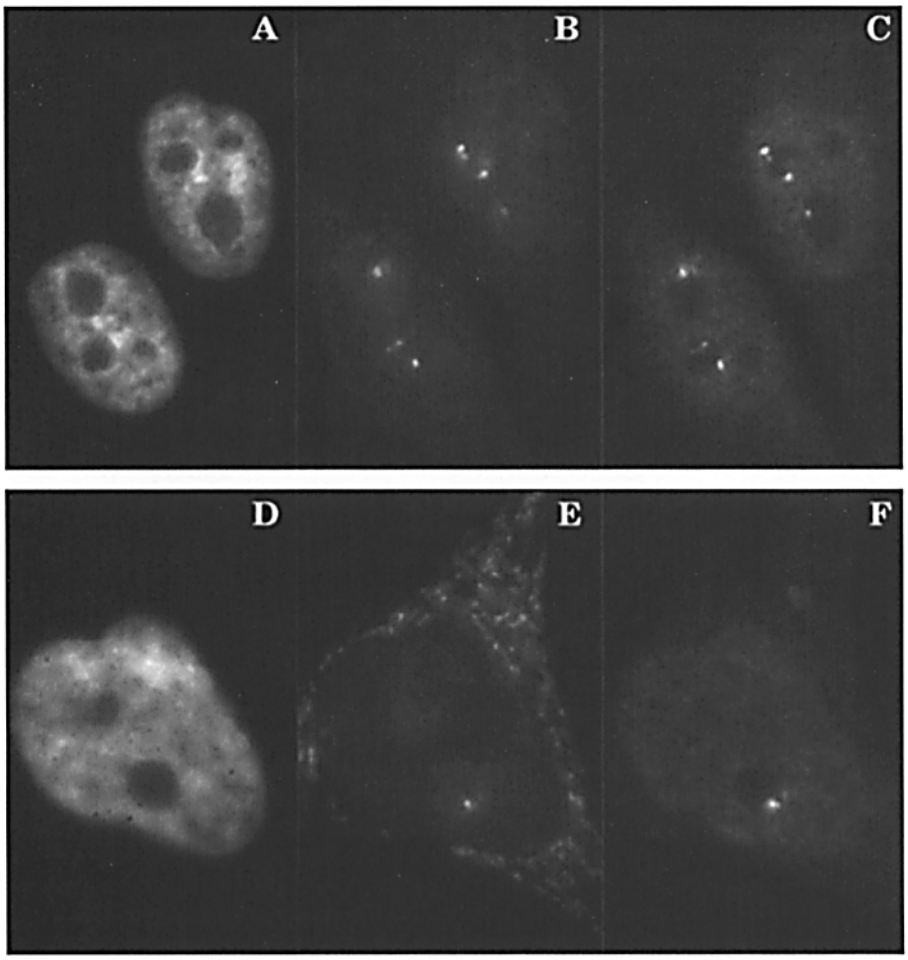

Figure 7. The polypyrimidine tract-binding protein (hnRNP I) is concentrated in PNCs. HeLa cells post-extracted with either acetone (*A–C*) or Triton (*D–F*) were hybridized with (*B*) hY1(71-82) or (*E*) hY5(41-52) and then incubated with anti-hnRNP I mAb 7G12 (*C* and *F*). Nuclei were counterstained in DAPI (*A* and *D*). Prolonged incubation in the relatively low ionic strength of the antibody dilution buffer (PBS-A) occasionally reduced the intensity of the cytoplasmic fluorescence, presumably due to dissociation of the hybridized oligomer (*B*). However, it is clear that the PNCs visualized with hY oligomers colocalize with the perinucleolar structures detected with the anti-hnRNP I mAb (compare *B* with *C* and *E* with *F*).

ing experiments. These experiments revealed that the PNCs labeled by anti-hY oligomers are identical to the perinucleolar structures that contain hnRNP I/PTB (Fig. 7, *A–F*).

Discussion

Structures of Ro RNPs

Our experiments, using oligonucleotide-directed RNase H cleavage and 2′-OMe oligonucleotides, have allowed us to identify regions of Y RNAs that are accessible, within native Ro RNPs, for interactions with other RNAs or proteins. The results demonstrate that the pyrimidine rich internal loops predicted to form in all Y RNAs are accessible for base-pairing with exogenous oligonucleotides. As many small RNPs interact with other RNA molecules via base pairing, these loops could be involved in such interactions.

Recently, the structures of in vitro–synthesized hY1 and hY5 RNAs were determined using both chemical modification and enzymatic probing techniques (van Gelder et al., 1994). In these experiments, the large pyrimidine-rich internal loop in Y1 was not accessible using either technique. As a result, this portion of the loop was proposed to either participate in tertiary interactions or to form an unusual structure. The fact that this region of the loop is accessible to oligonucleotides when the RNA is in the RNP form suggests that the loop may also be accessible in vivo.

Although the subcellular location of Ro RNPs was controversial for many years (Hendrick et al., 1981; Harmon et al., 1984), it was recently shown, using fractionation procedures in which nuclear leakage is minimized, that the RNPs are predominantly cytoplasmic (O'Brien et al., 1993; Peek et al., 1993). Our in situ hybridization data are consistent with this, as we estimate that >90% of the hybridization signal is cytoplasmic when the oligonucleotides are used at 0.2 pmol/μl. The nuclear signals seen when the hY1 and hY3 oligomers are used at higher concentrations may therefore represent nonspecific binding to other structures, as a large nuclear pool of Y RNPs was not detected in karyoplasts prepared from HeLa cells (Peek et al., 1993).

A Novel Perinucleolar Compartment

Using oligonucleotides directed against accessible regions of Y RNAs, we have identified a nuclear subdomain in which certain polymerase III transcripts appear to accumulate. This PNC is identical to the perinucleolar structure containing the polypyrimidine tract binding protein, hnRNP I/PTB, that was previously described by Ghetti et al. (1992). This structure is distinct from coiled bodies and does not correspond to the site of transcription of the Y

RNAs or 5S rRNA. In addition to three of the four human Y RNAs and hnRNP I/PTB, PNCs also appear to contain a fraction of the RNase MRP and RNase P RNAs. These results suggest that PNCs represent a previously undescribed nuclear subcompartment which is involved in some aspect of the metabolism of polymerase III transcripts.

Although we have performed the majority of our experiments in HeLa cells, we have also detected PNCs in several other human cell lines. While the percentage of cells exhibiting PNCs is highest in HeLa cells, we have observed PNCs in ~20–50% of 293, HT-1080 and HEp-2 cells (unpublished data). However, we also observed that fewer than 10% of WI-38, IMR-90, and Detroit 551 cells displayed PNCs. Thus, PNCs are similar to coiled bodies in being readily detectable in highly transformed cell lines, less easily detectable in immortalized (but untransformed) lines and barely detectable in cells of defined passage number (Spector et al., 1992)

Curiously, while the hY3(65-76) oligomer detects PNCs, two other 2′-OMe oligomers directed against overlapping portions of the hY3 loop fail to stain PNCs. One possible explanation is that the oligonucleotides that do not hybridize to PNCs are directed against portions of the loop that are inaccessible when hY3 RNA is located in PNCs, perhaps because the loop is bound by protein. However, the observation that certain oligonucleotides that efficiently select hY3 RNA from extracts do not concentrate in PNCs suggests that our other negative results, such as the failure to detect 7SL RNA and 5S RNA in PNCs, must be interpreted with caution.

Because the most accessible region of each Y RNA is the large pyrimidine-rich internal loop, most of the oligonucleotides that detect PNCs tend to be purine-rich. It is therefore possible that these oligonucleotides are hybridizing to other RNAs, such as ribosomal RNA or to a heterogeneous pool of mRNPs, located within PNCs. We think this is unlikely for the following reasons. First, although all the hY oligonucleotides that detect PNCs are purine-rich, certain oligonucleotides that do not detect PNCs, such as hY3(57-70), are equally rich in purines. Second, the oligonucleotides that hybridize to MRP RNA and RNase P RNA have more standard base compositions, yet also detect PNCs. However, definitive proof that these five RNAs are actually located within PNCs will require purification of these structures.

Although we have detected high concentrations of five polymerase III RNAs in PNCs, the proteins that normally bind these RNAs are not enriched within this compartment. Since all the Y RNAs found in PNCs contain a pyrimidine-rich internal loop, it is possible that the RNAs in PNCs are bound by hnRNP I/PTB, which has been cross-linked to polypyrimidine tracts. Intriguingly, the hY4 RNA loop does not contain a run of pyrimidines and this RNA is not detected in PNCs. Using antibodies against hnRNP I/PTB in immunoprecipitation experiments, we have been unable to detect an interaction between PTB and the Y RNAs. However, if hnRNP I/PTB interacts transiently with Y RNAs, or if the interaction is a low affinity one, it might not be detectable by immunoprecipitation.

In some ways the PNCs may be analogous to coiled bodies, which contain high concentrations of the spliceosomal U snRNAs, U1, U2, U4, U5, and U6 (Lamond and Carmo-Fonseca, 1993; Gall et al., 1995). In addition, a nucleolar snRNA, U3, was recently detected in HeLa cell coiled bodies (Jimenez-Garcia et al., 1994). In Xenopus egg extracts, coiled bodies assembled in vitro have been shown to contain the spliceosomal U snRNAs, the U3 and the U8 small nucleolar RNAs (Bauer et al., 1994). U7 snRNA has also been detected within the coiled bodies (C snurposomes) of *Xenopus*, human and mouse cells (Wu and Gall, 1993; Frey and Matera, 1995). Because they contain so many snRNAs with distinct functions, it has been proposed that they represent a temporary place of assembly for snRNPs after their import into nuclei (Bauer et al., 1994). Similar to coiled bodies, PNCs contain several RNAs with distinct functions. However, while the small RNAs detected in coiled bodies are largely transcribed by RNA polymerase II, the RNAs detected in PNCs are all transcribed by RNA polymerase III.

The observation that PNCs contain several different small RNAs suggests that, like coiled bodies, they may represent cellular compartments in which some aspect of RNP biogenesis or assembly occurs. As PNCs do not coincide with the interphase position of the Y1 and Y3 or 5S genes, they are unlikely to represent sites of RNA transcription. Our failure to detect the La protein in PNCs also suggests that the RNAs in PNCs are not nascent polymerase III transcripts. One possibility is that PNCs are staging areas for newly synthesized RNAs that are destined for the cytoplasm. Alternatively, the RNAs detected in PNCs might shuttle between the nucleus and cytoplasm. Of particular importance is that like several other hnRNP proteins, hnRNP I is also known to shuttle (Pinol-Roma and Dreyfuss, 1992; Dreyfuss, G., personal communication). In this scenario, PNCs might represent the place at which shuttling RNAs are assembled and sorted after entry into the nucleus or before export to the cytoplasm.

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