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Discrete Nuclear Domains of Poly(A) RNA and Their Relationship to the Functional Organization of the Nucleus

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Abstract. The functional organization of the nucleus was studied using a fluorescence microscopy approach which allowed integration of positional information for RNA, DNA, and proteins. In cells from sea urchin to human, nuclear poly(A) RNA was found concentrated primarily within several discrete "transcript domains" which often surrounded nucleoli. Concentrations of poly(A) RNA were coincident with snRNP antigen clusters, providing evidence for the localization of pre-mRNA splicing at these sites. The spatial relationship of transcript domains with respect to various classes of DNA was established, in that the poly(A) RNA-rich regions coincided with discrete regions of low DNA density and were non-randomly distributed with respect

to specific DNA sequences. Centromeric DNA and late-replicating DNA did not overlap transcript domains, whereas a subset of early-replicating DNA may. Results indicate that transcript domains do not result directly from a simple clustering of chromatin corresponding to metaphase chromosomes bands. Finally, observations on the reassembly of these domains after mitosis suggest that the clustering of snRNP antigens may be dependent on the reappearance of pol II transcription. Implications of these findings for overall nuclear structure and function are considered, including a discussion of whether transcript domains may be sites of polymerase II transcription reflecting a clustering of active genes.

THE cell nucleus performs numerous complex tasks which include packaging the enormous length of DNA, high fidelity replication of this DNA, transcription, processing, and transport of different classes of RNA, protein import and export, and precise redistribution of nuclear components during mitosis. Additionally, many of these are done in a cell-type specific manner. It has often been proposed that the extremely dense structure of the nucleus may be spatially compartmentalized as a means to efficiently carry out its multiple functions (see Comings, 1968, 1980). However, evidence for such physical partitioning has been modest. For example, while great advances have been made in understanding the biochemical steps involved in pre-mRNA transcription and processing, the subnuclear location of these events and the extent to which they are compartmentalized is unknown. In this paper we investigate the potential compartmentalization of these and other nuclear events by analyzing the distribution of poly(A) RNA in relation to several nuclear constituents of known functional significance.

Unlike the cytoplasm, there are no lipid membranes in the nucleus, but the confinement to the nucleolus of rRNA genes originating on several different chromosomes shows that elegant spatial and functional regionalization is possible. Strong evidence supporting the early suggestions that interphase centromeres and telomeres are specifically positioned (Rabl, 1885) and individual chromosomes occupy distinct territories (Boveri, 1909) has been presented recently by several

laboratories (Reviewed in Hadlaczy et al., 1986; Manuelidis, 1990; Haas and Schmid, 1991). While most studies have focused on the position of entire chromosomes or abundant non-expressed sequences, visualization of a single gene and its cognate RNA suggests that the interphase positions of specific active sequences and their primary transcripts may also be highly localized (Lawrence et al., 1988; Lawrence et al., 1989). That interphase chromatin may be functionally as well as spatially compartmentalized is also implied by the fact that metaphase chromosomes display unique and highly reproducible patterns of light and dark bands with respect to which genes (reviewed in Bickmore and Sumner, 1989), replicating DNA (Ganner and Evans, 1971; Holmquist et al., 1982), repetitive sequences (Manuelidis and Ward, 1984; Korenberg and Rykowski, 1988), nuclease sensitivity (Gazit, 1982; Kerem, 1984), and certain proteins (Disney et al., 1989) are specifically positioned. It is quite possible that these bands correspond to some distinct functional partitioning of chromatin at interphase.

Additional evidence for higher level nuclear organization comes from immunolocalization studies which established early on that nuclear antigens recognized by serum from autoimmune disease patients can be categorized as either homogeneous, nucleolar, or "speckled" (Beck, 1961). Despite extensive investigation of autoimmune antigens, the reasons for many of them having nonhomogeneous nuclear localization are not known. Some autoimmune antibodies selectively precipitate small nuclear ribonucleoprotein particles

(snRNP)¹ involved in pre-mRNA splicing (Lerner et al., 1979, 1980, 1981; Yang et al., 1981). Although not universally accepted, there is evidence that antigens from this class of snRNPs are clustered primarily in certain nuclear regions in mammalian somatic cells (Mattioli and Reichlin, 1971; Northway and Tan, 1972; Lerner et al., 1981; Deng et al., 1981; Tan, 1982; Spector et al., 1983; Fakan et al., 1984; Reuter et al., 1984; Smith et al., 1985; Nyman et al., 1986; Ringertz et al., 1986; Spector, 1990; Zieve and Sauterer, 1990) and in the sphere organelle in amphibian oocytes (Gall and Callan, 1986; Wu et al., 1991). A spliceosome assembly factor also localizes to these areas (Fu and Maniatis, 1990), but it remains to be shown unequivocally whether these regions exist *in vivo*, and if so whether they are sites of pre-mRNA processing or, alternatively, sites of snRNP assembly or storage, with pre-mRNA processing occurring elsewhere (Meadows, 1990; Fu and Maniatis, 1990; Spector, 1990; Zieve and Sauterer, 1990).

A well-integrated structural and functional view of the nucleus will require approaches which simultaneously localize, with high resolution, specific functionally distinct nuclear constituents, and which ultimately consider the distribution of nuclear RNA as well as DNA and proteins. Using such an approach we have studied the intranuclear distribution of poly(A) RNA because of its fundamental significance for understanding the spatial organization of pre-mRNA transcription, processing, and transport. Approximately 90% of mRNA is polyadenylated and essentially all nuclear poly(A) sequence occurs as 3' tails on hnRNA destined to become cytoplasmic message (reviewed in Lewin, 1975; Puckett and Darnell, 1976; Brawerman, 1981; Nevins, 1983), therefore the distribution of this broad category of RNAs can be investigated by fluorescent *in situ* hybridization to their poly(A) sequences. Taking this strategy we demonstrate that poly(A) RNA is concentrated in discrete domains within the nucleus. The positions of these "transcript domains" were compared in individual cells with locations of the nucleolus, total DNA, replicating DNA, centromeric DNA, and RNA processing components. The potential relationship of this distinct nuclear compartment to chromosome organization was also considered as was the reassembly of these areas following mitosis.

Materials and Methods

Cell Culture

Human diploid fibroblasts, originally cultured from the foreskin of a normal male newborn (Folkman and Haudenschild, 1980) were grown as monolayers in DME plus 0.1% glucose and 10% FCS (Gibco Laboratories, Grand Island, NY). Mink lung epithelial line ATCC CCL 64 and human intestinal smooth muscle line ATCC CRL 1692 were grown similarly according to recommendations of the American Type Culture Collection (Rockville, MD).

Cell Fixation

Unless otherwise noted cells were fixed as follows: coverslips with attached cells were rinsed two times with PBS, pH 7.4, at room temperature, and then incubated on ice for 20–30 s in succession in PBS, CSK buffer (100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 10 mM PIPES, pH 6.8; Fey

1. **Abbreviations used in this paper:** BrdU, bromodeoxyuridine; HDF, human diploid fibroblast; Pol I-II, polymerase I-II; snRNP, small nuclear ribonucleoprotein particles.

et al., 1986), CSK buffer plus 0.5% triton X-100, and again in CSK buffer. The RNase inhibitor vanadyl adenosine (2 mM) was added to each of these buffers just before use. Cells were immediately fixed for 10 min at room temperature in 4% paraformaldehyde in PBS, pH 7.4, and stored in 70% ethanol at 4°C until use. Other work has shown that this treatment does not affect the spatial or quantitative retention of specific RNAs (Xing and Lawrence, 1991) and that >95% of poly(A) is retained throughout these procedures (Taneja and Singer, manuscript in preparation). The nuclear signal pattern was essentially identical in triton-treated and non-triton-treated cells, however, nuclear signal in nontriton-treated was somewhat reduced unless nuclei were permeabilized by prolonged ethanol storage.

While the pattern of transcript domains/snRNP clusters varied somewhat with different fixation protocols, as noted by others (Carmo-Fonseca et al., 1991), the presence of discrete nuclear domains in our experiments was consistently correlated with high-quality fixation, based on cell morphology, total RNA retention (judged by fluorescent RNA stains such as propidium iodide and acridine orange), and retention of DNA in the nucleus (based on DAPI staining). Signs of poor fixation such as large amounts of DNA looping out of the nucleus following *in situ* hybridization were invariably accompanied by more homogeneous poly(A) RNA patterns. Likewise, storage of fixed cells in PBS rather than ethanol resulted in the loss of RNA, and possibly other nuclear constituents (Lawrence et al., 1985) and less discrete localization of poly(A) RNA. Paraformaldehyde fixation followed by ethanol storage consistently resulted in both high quality fixation followed by ethanol storage consistently resulted in both high quality fixation and detection of discrete nuclear clusters of poly(A) RNA and snRNPs.

Alternative fixation methods used included: 4% and 1% paraformaldehyde for 10 min; 20:1 or 3:1 methanol/acetic acid for 2 h followed by 1 h of ethanol; acetone for 30 min; -20°C ethanol 2 h; and baking for 2 h at 65°C.

In Situ Hybridization

Hybridization and fluorescence detection were based on previously developed methods described in detail elsewhere (Lawrence and Singer, 1985; Lawrence et al., 1988, 1989; Johnson et al., 1991b). Oligo dT₅₅ (T₅₅) was end labeled with biotin-16-dUTP (Bethesda Research Laboratories, Gaithersburg, MD) and purified using a G-50 Sephadex column (Taneja and Singer, in preparation). Hybridizations were done at 37°C in our standard buffer and 15% formamide (Johnson et al., 1991b). Detection was done using fluorochrome-conjugated avidin (Enzo Biochemical) in 4× SSC.

Hybridization Controls

To establish that T₅₅ signal resulted from specific hybridization, parallel experiments were done replacing oligo dT with dA (see Fig. 1), dC, or dG (not shown), each of which resulted in no signal. When cells were hybridized with biotin- or ³²P-labeled T₅₅ followed by subsequent washes at stepwise increases of 5°C (starting at 35°C), >95% of the signal was lost between 45 and 50°C, indicative of the sharp melting curve of oligonucleotide hybrids. Excess nonlabeled T₅₅ or A₅₅, but not a random sequence 55 mer, inhibited binding of labeled T₅₅. When fixed cells were treated before hybridization with 0.2 N NaOH to remove cellular RNA, no T₅₅ hybridization occurred. This was not because of inhibition of hybridization or detection since centromeric DNA was readily detectable under identical conditions. Likewise snRNP antigens remained intact during NaOH treatment, as determined by immunofluorescence, further suggesting that the loss of T₅₅ signal was due specifically to the removal of RNA.

Areas of low T₅₅ signal, for example near the nuclear envelope and in early G₁ cells, do not result from low accessibility to probe molecules since centromeres were readily detected here.

Immunofluorescent Staining of snRNP Antigens

The snRNP mAb used, Y12, is categorized as an anti-SM Ab because it selectively precipitates ribonucleoprotein complexes containing U1, U2, U4, U5, and U6 RNAs, all of which are involved in pre-mRNA splicing (Lerner and Steitz, 1979; Lerner et al., 1981; Petterson et al., 1984; Ringertz et al., 1986; Zieve and Sauterer, 1990 for review). Cells were treated with Y12 in PBS, 1% BSA at 37°C for 45 min (staining in 4× SSC also worked well) and detected using a rhodamine-conjugated goat anti-mouse Ab (Cappel Laboratories, Malvern, PA). For simultaneous detection of poly(A) RNA and snRNPs, T₅₅ was hybridized and detected as above except that anti-snRNP Ab was added during the biotin detection step and was subsequently visualized using a secondary Ab.

Table I. Chromosome 17 Centromere Location

Position relative to transcript domains	Investigator number 1	Investigator number 2
In plane coincident	<0.01%	0
In plane bordering	5	3
In plane completely separate	27	30
Out of plane	67	67

Analyses were done on several hundred cells, independently, by the two investigators. For details see Fig. 5 and "Microscopy" in Materials and Methods.

Microscopy

Microscopes (Zeiss, Oberkochen, Germany) equipped with epifluorescent filters were used. All pictures were taken with standard 35-mm film and have not been "image processed." Many of the analyses, particularly those requiring judgements about the position of signals in the Z axis (For example, see Figs. 5, 6, and Table I), were done with a low depth-of-field ($\sim 0.5 \mu\text{m}$), 100 \times , neofluor objective. Thus, in a typical nucleus a few microns thick, several focal planes could be independently analyzed.

Poly(A) RNA, Centromeric DNA Double Label

Poly(A) RNA was hybridized in paraformaldehyde-fixed cells and detected with avidin as described above. Cells were then re-fixed for 10 min in 4% paraformaldehyde and digoxigenin-labeled centromeric DNA probe was hybridized following denaturation of cellular DNA in 70% formamide 2 \times SSC as described elsewhere (Johnson et al., 1991b).

Poly(A) RNA, Replicating DNA Double Label

To label replicating DNA in all stages of S-phase, cells in non-synchronous cultures were treated with 25 $\mu\text{g/ml}$ bromodeoxyuridine (BrdU) for 15 min

before fixation. Poly(A) RNA was labeled and cells were re-fixed as described in the previous section. Cells were then treated for 10 min in 4N HCl before detection of BrdU with a fluorochrome-conjugated anti-BrdU antibody (Boehringer Mannheim Biochemicals, Indianapolis, IN).

Results

The approach reported here is based on previous methodological studies which identified and optimized conditions for the preservation and detection of DNA and RNA by in situ hybridization (Lawrence and Singer, 1985; Lawrence et al., 1989; reviewed in Lawrence, 1990). Our goal was to provide an accurate overview of the relative distributions of several different nuclear constituents in intact cells, therefore we chose to use standard two-dimensional fluorescence microscopy which made it possible to base conclusions on the analyses of thousands of cells in dozens of experiments. All photographs presented are unprocessed images directly as they appear through the microscope. Computer-assisted reconstructions providing more detailed three-dimensional information based on analysis of a few cells will be presented elsewhere (Carter, K. C., F. Fay, and J. B. Lawrence, manuscript in preparation).

Polyadenylated RNA Is Concentrated in Discrete Nuclear Domains

In initial studies, using paraformaldehyde-fixed human diploid fibroblasts (HDF), hybridization with biotinated oligo-dT₅₅ (T₅₅) probe resulted in intense fluorescent signal which localized primarily to several discrete regions of each interphase nucleus (Fig. 1, A and B). Various control experiments indicated that these discrete regions represent genuine hybridization to poly(A) RNA fixed in a state which reflects its

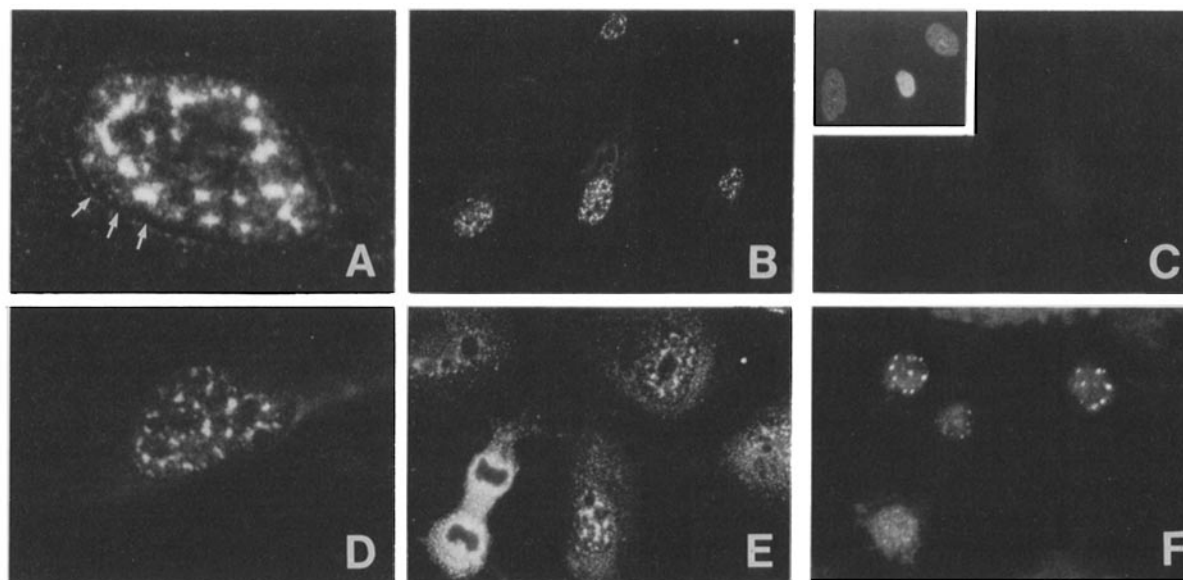


Figure 1. Distribution of Poly(A) RNA within various cell types. (A) Typical nucleus of a human diploid fibroblast (HDF) hybridized with biotinated T₅₅ and detected with fluorescein-conjugated avidin. Note discrete, brightly staining regions (transcript domains) as well as the dim signal throughout the nucleus. Also note the area of low signal just inside the nuclear envelope (arrows). This region is clearly inside the nucleus as shown by counter staining with DNA dyes (see Fig. 3 A). (B) Low magnification view of poly(A) RNA in HDFs. (C) HDFs hybridized as in (A), but substituting A₅₅ for T₅₅ resulting in no observable signal. (Inset) Location of nuclei as shown by DAPI staining. (D-F) Poly(A) RNA distribution in human intestinal smooth muscle cells (D); Mink lung epithelial cells (E); note telophase cell at lower left); and sea urchin coelomocytes (F).

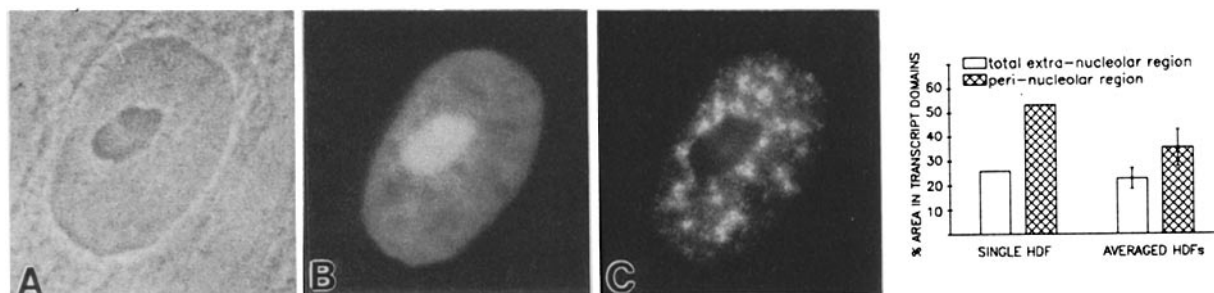


Figure 2. Distribution of transcript domains in relation to the nucleolus. The nucleus of a single HDF is shown by phase contrast (*A*); counterstaining with propidium iodide (*B*); and fluorescence detection of poly(A) RNA (*C*). Note that the nucleolus, which is clearly seen as a discrete dark region under phase contrast (*A*), is brightly staining with propidium iodide due to the presence of double stranded rRNA (*B*) and devoid of poly(A) RNA signal (*C*). Note the accumulation of transcript domains around the nucleolus. (*Chart*) Percent of area occupied by transcript domains in the total non-nucleolar nuclear area (\square) and the area within $\sim 2 \mu\text{m}$ of the nucleolus (\otimes). Data are shown for cell at left and for 11 randomly chosen nuclei containing a single nucleolus (mean \pm SD). An increase in the average space occupied by transcript domains around the nucleolus was seen in every cell measured and was as high as twofold, as in the cell shown.

in vivo distribution. For example, discrete regions of T₅₅ signal occurred using various fixation conditions (see Fig. 3 *A*), but hybridization using a control oligo-dA₅₅ probe resulted in no signal (Fig. 1 *C*). Additional control experiments are described in Materials and Methods. In repeated experiments we consistently observed that total nuclear poly(A) RNA was not uniformly or randomly distributed, but was concentrated primarily in a relatively small number of distinct sites. Additionally there was often an area of high poly(A) RNA concentration just outside the nucleus, as well as signal more diffusely distributed throughout the cytoplasm to be described in detail elsewhere (Taneja and Singer, manuscript in preparation).

There were approximately 10 to 20 distinctly bordered signal clusters in most nuclei and each cluster ranged from less than one micron to a few microns in diameter. Relative to the signal intensity from single-copy sequences of known size, the dimensions of these regions and the intensity of the signal indicate a minimum of many thousands of molecules per cluster, as confirmed by quantitative microfluorimetry (Carter et al., manuscript in preparation). Most clusters were roughly spherical and did not appear to interconnect with other poly(A) RNA regions on any focal plane; in a few cells there were longer multi-lobed regions. In many cells poly(A) RNA regions seemed to ring nucleoli, but were not seen inside nucleoli. Outside the concentrated poly(A) RNA regions, or transcript domains, there was also faint signal

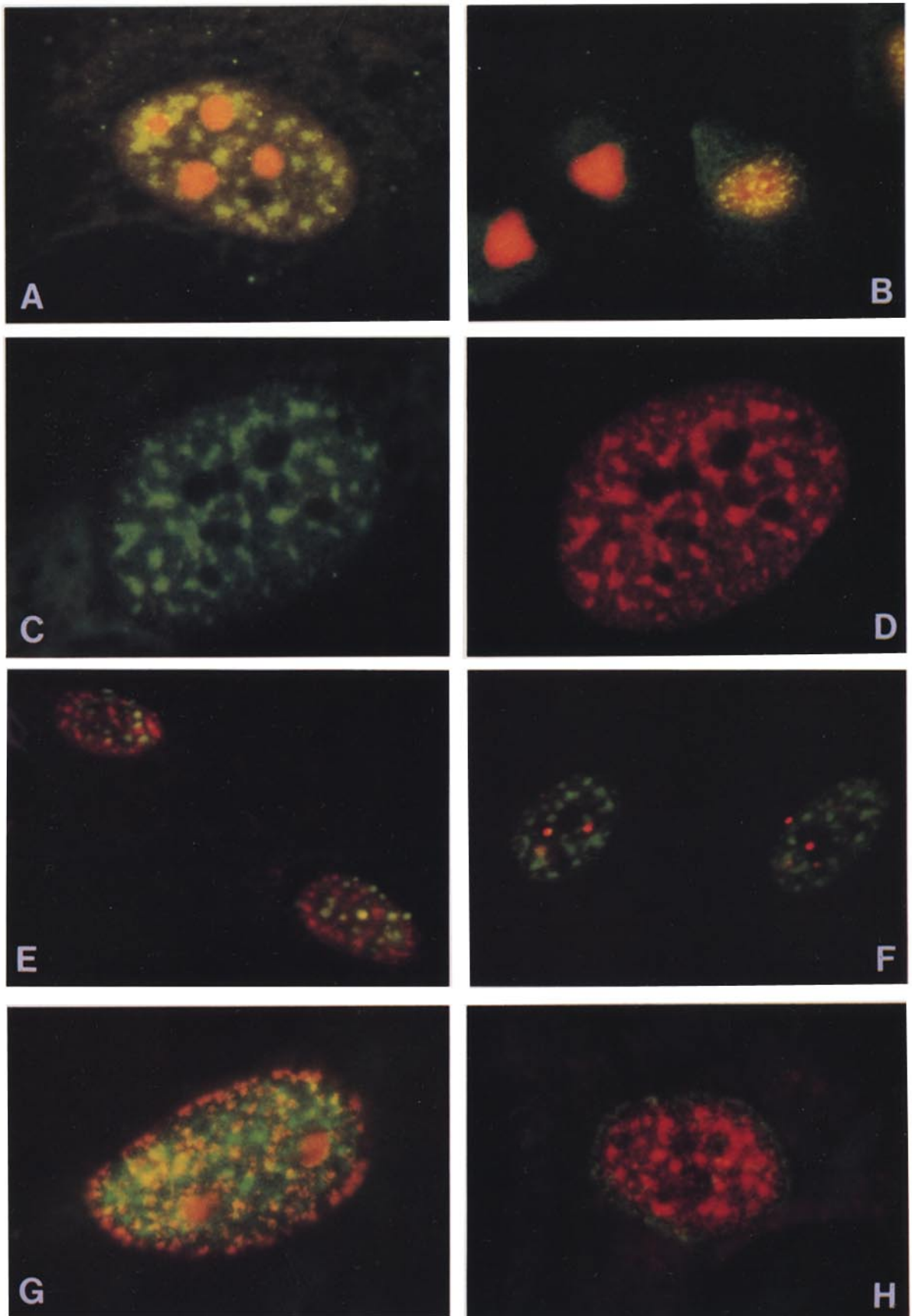
throughout the nucleus, except in the nucleolus (Figs. 1 *A* and 2), and a region of markedly low signal just inside the nuclear envelope $\sim 1 \mu\text{m}$ wide (Fig. 1 *A* and 3 *A*).

To ask whether nuclear transcript domains occur in other species and cell types, we visualized poly(A) RNA in 26 cell lines and primary cultures from human, rat, mouse, chicken, mink, and sea urchin (Fig. 1, *D-F*). In each case poly(A) RNA was concentrated in distinct nuclear regions. Furthermore, the encircling of nucleoli with transcript domains was frequently observed and quantitative analysis indicated nucleolar association in HDFs was significantly greater than would be predicted by random distribution of transcript domains (Fig. 2).

Transcript Domains Coincide with Clusters of snRNP Antigens

Messenger RNA is formed through a complex series of steps which includes transcription, 5' end capping, 3' end cleavage, addition of a 3' poly(A) tail, removal of introns, and transport of the mature mRNA to the cytoplasm. It is not known whether any of these steps are physically compartmentalized within the nucleus. There is evidence that transcription and polyadenylation are closely linked (see Discussion), and we reasoned that areas of high poly(A) RNA concentration might be sites of either pre-mRNA transcription, one or more processing steps, a rate limiting step in transport, or

Figure 3. Simultaneous detection of poly(A) RNA and other constituents of the nucleus in HDFs. All photographs are single exposure unprocessed images taken using a standard fluorescence microscope. *E-H* were taken using a dual wave-length filter set which allows simultaneous visualization of different fluorochromes in precise registration with no optical shift (Omega Corp., Brattleboro, VT; Johnson et al., 1991a). (*A* and *B*) Simultaneous visualization of poly(A) RNA (*green*) and propidium iodide counterstain (*red*) using a wide-pass fluorescence filter set. Note the area along the nuclear rim in *A* which is devoid of poly(A) RNA signal. Cell in *A* was fixed in acetone. *B* shows a pair of recently divided daughter cells which were easily identified by cell morphology and because they had no nuclear poly(A) signal, in striking contrast to all other cells in the vicinity (also see Fig. 7). (*C* and *D*) Poly(A) RNA (*C*) and snRNP antigens (*D*). Note the precise coincidence in spatial distribution of the two signals in the nucleus, particularly in the clustered regions. Also note cytoplasmic signal for poly(A) but not snRNP. (*E*) Poly(A) RNA (*red*) and centromeric DNA (*yellow*) under conditions which detect all centromeres. (*F*) Poly(A) RNA (*green*) and chromosome 17 centromere (*red*). Note the extreme peripheral position of one centromere in the nucleus to the right. (*G*) Poly(A) RNA (*green*) and replicating DNA (*red*). (*H*) Poly(A) RNA (*red*) and replicating DNA (*green*). Note the presence of replicating DNA in the area of low poly(A) RNA signal along nuclear periphery in both *G* and *H*. Areas which appear yellow in the photographic print in *G* represent signal from patches of replicating DNA which were in focal planes above the poly(A) regions and were not actually overlapping.



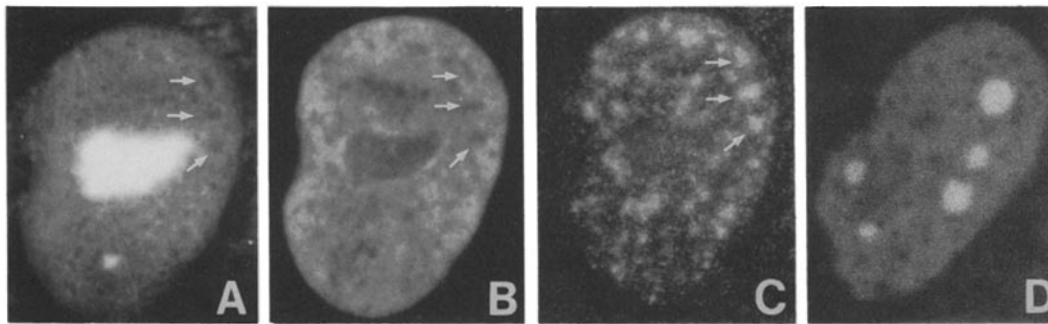


Figure 4. Detection of low density DNA areas corresponding to poly(A) transcript domains. A single HDF nucleus is shown, comparing the distributions of propidium iodide (A), and DAPI (B), with poly(A) RNA (C). Arrows are for orientation and to denote typical areas of low DNA which correspond to transcript domains. (D) Nucleus of an unextracted, unfixed HDF grown in the presence of propidium iodide for 30 min. Similar distributions were seen in cells extracted with triton but not fixed before staining.

all of the above. Hence, experiments were undertaken to address whether any relationship could be discerned between the distribution of poly(A) RNA and snRNP antigens involved in splicing.

Double-label experiments, using an antibody specific to snRNPs of the pre-mRNA splicing class, revealed a strong coincidence between poly(A) RNA regions and snRNP antigen clusters (Fig. 3, C and D). This was repeated several times and analyzed using a dual-wavelength filter set which makes possible highly precise comparisons of different wavelength fluorescence distributions. With the exception of recently divided early G₁ cells (see below), experiments which gave high detection efficiency for both poly(A) RNA and snRNP antigens consistently resulted in virtually complete overlap of the nuclear immunofluorescence signals. The colocalization of poly(A) RNA and snRNP antigens indicates that transcript domains are not solely transcription or transport sites and that snRNP protein clusters are not simply areas of snRNP assembly or storage. This co-localization supports the interpretation that these regions are sites of pre-mRNA processing.

Organization of Poly(A) RNA with Respect to the Underlying Genome

Regions of markedly concentrated poly(A) RNA may form to facilitate the processing and/or transport of RNA in a manner independent of the underlying chromatin. Alternatively, a greater degree of functional organization would be implicated if the RNA was specifically positioned with respect to DNA. Hence, to understand the organizational underpinning of the nucleus it is important to address whether this major class of RNA is organized with respect to DNA. We investigated this at the level of DNA condensation, DNA sequence, and DNA replication.

Staining of interphase DNA with various fluorescent dyes revealed a nonhomogeneous distribution of chromatin. Discrete regions of low DNA density or condensation consistently occurred in fixed cells stained with either DAPI, which is DNA specific, or propidium iodide, which binds double-stranded DNA or RNA. These areas corresponded strikingly with transcript domains in all of several cell types examined (Fig. 4). Interestingly, a more defined border consistently occurred around these areas using propidium iodide as opposed to DAPI. Unlike the virtually complete overlap between snRNP antigens and poly(A) RNA, in some cells

there was a small number of low DNA density areas which did not correspond to transcript domains. The use of DNA stains to identify transcript domains provided a way to confirm whether they exist *in vivo*. Staining DNA in living cells revealed areas of low DNA concentration similar in size, distribution, and number to those seen in fixed nuclei (Fig. 4 D). This strongly supports the relevance of these structures *in vivo*.

We further explored the relationship of RNA distribution to that of the underlying genome by simultaneously visualizing poly(A) RNA and centromeric DNA to address two major questions: Is there a random or non-random distribution of specific genomic sequences relative to transcript domains? Is transcriptionally inactive DNA included or excluded from these regions? The DNA probe used hybridizes to a non-transcribed, tandemly repeated satellite sequence on all centromeres under low stringency conditions, or preferentially to the centromere of chromosome 17 under high stringency conditions. In initial experiments, using both high and low stringency hybridization, it was immediately obvious that there was little or no overlap between centromeric DNA and poly(A) RNA regions (Fig. 3, E and F). For example, transcript domains were excluded from the area next to the nuclear envelope, whereas centromeric DNA was frequently present in this region (Fig. 3 F). As illustrated in Fig. 5 and quantitated in Table 1, of many hundred centromeric signals scored 97% were unambiguously separate from transcript domains.

These data clearly demonstrate non-random localization of poly(A) RNA regions with respect to DNA sequence. They further indicate that a nontranscribed satellite was totally excluded from these putative pre-mRNA processing areas. The more peripheral placement of centromeric DNA also supports the positioning of poly(A) RNA regions to interior portions of the nucleus.

Relating the Position of Transcript Domains at S-Phase to Subsets of Replicating DNA Which Form Bands on Metaphase Chromosomes

In further evaluating the relationship of transcript domains to overall nuclear structure, we investigated whether they exhibited any apparent spatial relationship with early or late replicating DNA, which distributes in clustered patterns within interphase nuclei (Nakayasu and Berezney, 1989). In addition, early and late subsets of replicating DNA generally

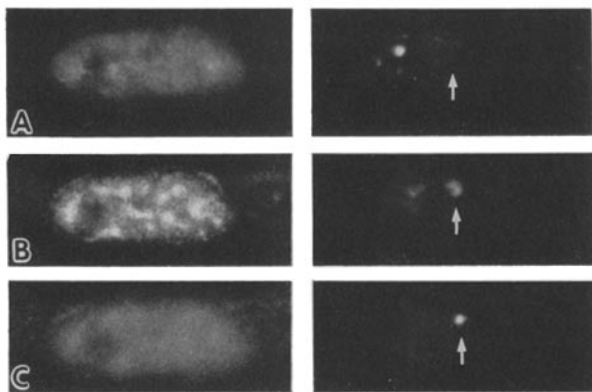


Figure 5. Demonstration of chromosome 17 centromeric DNA above and below poly(A) transcript domains in a single HDF nucleus. Using a low depth-of-field objective (see Materials and Methods) and standard photography, three focal planes are shown corresponding to the top (A), middle (B), and bottom (C) region of a nucleus. Signal for poly(A) RNA (left) and centromeric DNA (right) are shown. Arrows are for orientation. Note that one centromere is above regions of poly(A) RNA and the other is below, while out-of-focus light from both centromeres can be seen in the central plane.

correspond to transcriptionally active and inactive chromatin and localize to light and dark G-bands in metaphase chromosomes, respectively (reviewed in Bickmore and Sumner, 1989; Herbomel, 1990); hence, it becomes compelling to consider whether this non-homogeneous distribution of functional DNA classes, clearly visible at metaphase, is related to the clustered distribution of gene transcripts in interphase nuclei. Also, in light of current hypotheses which call for significant movement of DNA during replication (reviewed in Laskey et al., 1989), it was of interest to determine whether transcript domains remain intact throughout S-phase.

A two-color fluorescence protocol was developed which allowed simultaneous detection of poly(A) RNA and replicating DNA. Non-synchronous cultures were labeled for a short period (15 min) with BrdU, and many cells were analyzed so that the distribution of transcript domains could be compared with a full spectrum of different S-phase replication patterns (Fig. 6). The first thing these experiments demonstrated was that there is essentially no change in the general pattern of transcript domains during S-phase. An examination of hundreds of cells in multiple experiments showed that none of several replication patterns observed coincided with the pattern of poly(A) RNA regions. Hence, the transcript domains do not correspond in any obvious way to subsets of synchronously replicating DNA which become organized as bands on metaphase chromosomes.

While the overall patterns of poly(A) RNA and replicating DNA were clearly not the same, another question concerns whether there was any relationship or overlap between them. Several different patterns of replicating DNA were seen, and in many cells it was possible to identify patterns similar to those previously described for late- or early-replicating DNA (Nakayasu and Berezney, 1989). In most cells containing the clustered pattern characteristic of late-replicating DNA, poly(A) RNA regions and replicating DNA were consistently discernible as non-overlapping (Fig. 6 C). In contrast, in nuclei with the more finely distributed punctate pattern characteristic of early S-phase, during which most

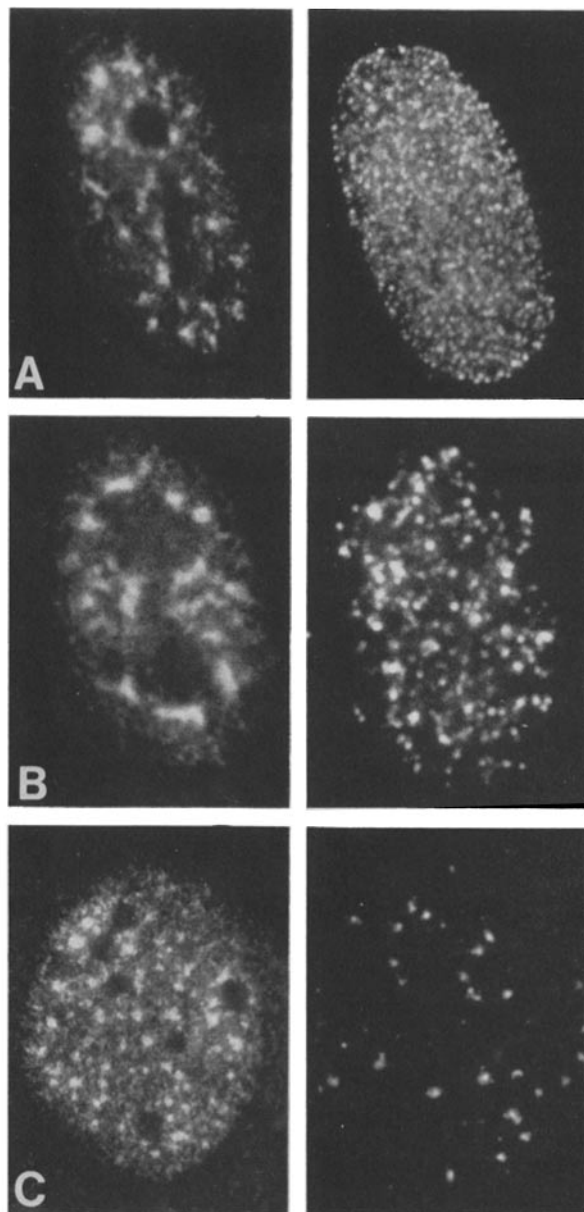
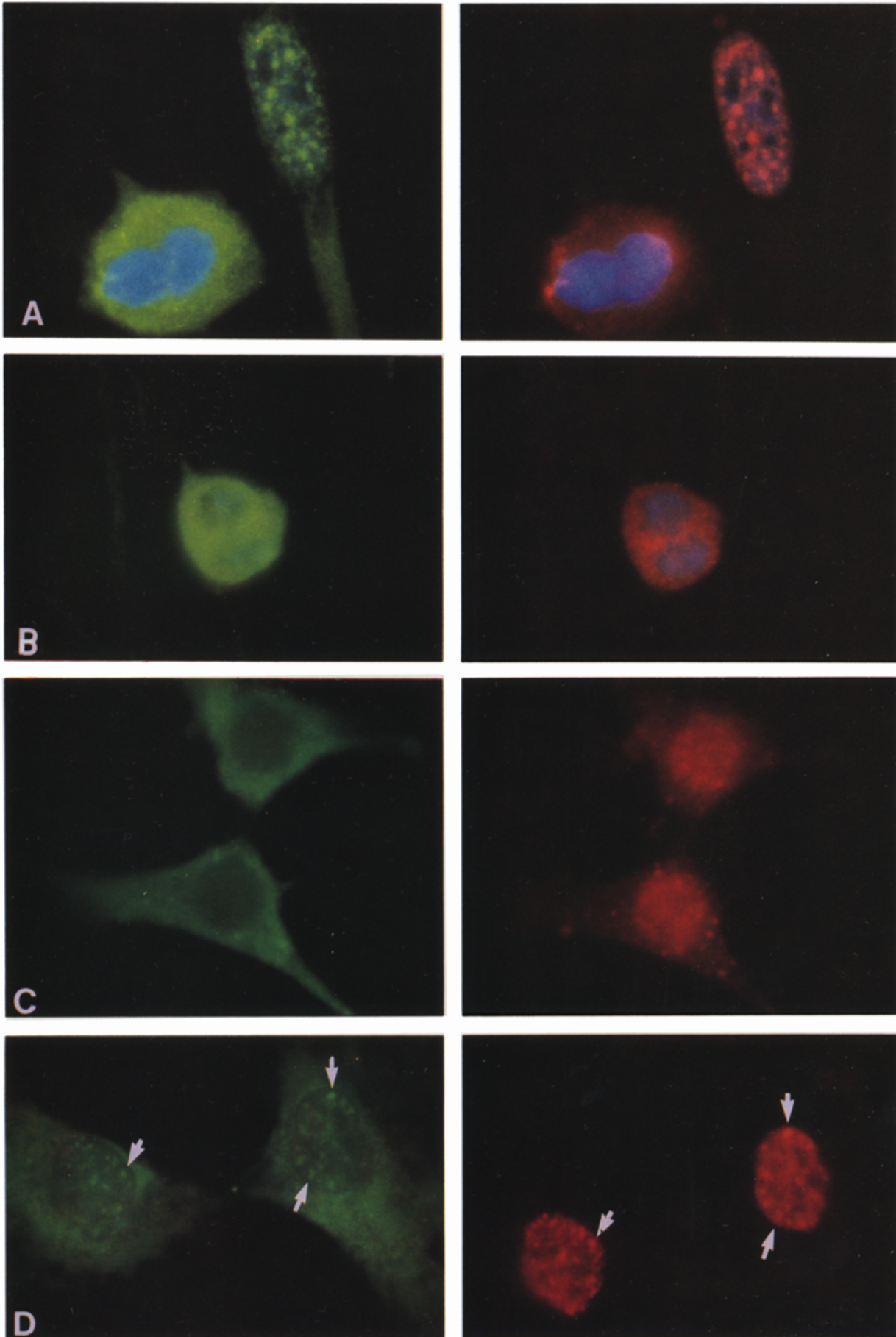


Figure 6. Simultaneous visualization of poly(A) RNA (left) and replicating DNA (right). Three of several replicating DNA patterns seen are shown (see text for details). Many cells had replicating DNA patterns which seemed to be typical of those previously described for early (A) or late (C) S-phase. Careful analysis with a low depth-of-field objective indicated that patterns like those in (B) and (C) had little or no overlap with transcript domains.

active genes replicate, some replicating DNA frequently appeared to overlap poly(A) RNA regions in the same focal plane (Fig. 6 A). This suggests that these regions may contain or be closely associated with subsets of early replicating DNA, however, further details will require in-depth three-dimensional analysis. In some cells, replicating DNA occupied the peripheral region near the nuclear envelope where poly(A) RNA concentration was markedly low, producing a striking ring of fluorescence (Fig. 3, G and H).

These data indicate that there is not a simple interphase coincidence between transcript domains and the functional subsets of DNA seen as metaphase bands. However, they do



suggest a spatial relationship. Furthermore, if splicing does occur in transcript domains, these results provide a clear demonstration of the differential nuclear compartmentalization of two distinct physiological processes, that of DNA replication and RNA processing.

Nascent Poly(A) Transcripts May Be Involved in the Reclustering of snRNPs following Mitosis

The existence of this distinct nuclear compartment, analogous to the nucleolus in that it may be responsible for processing of a major class of RNA, raises questions about the fundamental mechanisms which establish and maintain functional compartmentalization within the nucleus. Recently divided cells were readily apparent in our experiments (Fig. 1 *E*, and 3 *B*), hence, to study the process of assembling transcript domains during early G₁ we analyzed several hundred mitotic and postmitotic cells in preparations stained simultaneously for poly(A) RNA and snRNP antigens (Fig. 7). At metaphase, anaphase, and telophase, chromosomes were essentially devoid of both poly(A) RNA and snRNP although signal for each was seen throughout the cytoplasm. In telophase cells and newly divided daughter cells poly(A) RNA signal was often heavily concentrated just outside the nuclear envelope, but markedly absent inside the nucleus (Figs. 3 *B*, and 7 *C*). This indicates that even though poly(A) RNA concentration remains high during mitosis and surrounds mitotic chromosomes, this RNA is efficiently excluded from the nucleus as the nuclear envelope reforms.

In sharp contrast to other interphase cells, the early G₁ nuclear poly(A) RNA pattern was very different than that of snRNPs. In what appeared to be the earliest G₁ cells, snRNP antigens were seen in various stages of re-entry into the nucleus while nuclear poly(A) RNA remained undetectable. In these cells snRNP antigens had often re-entered the nucleus, but were distributed relatively uniformly or in a fine punctate pattern (Fig. 7 *C*). In apparently later daughter cells a dim poly(A) RNA signal was present in regions where snRNP antigens had begun to cluster (Fig. 7 *D*). In repeated experiments, poly(A) RNA was not seen in early G₁ nuclei in the absence of snRNP signal and snRNP proteins were not discretely clustered in the absence of poly(A) RNA signal. In addition, the discrete low density DNA areas, which correspond to transcript domains, appeared only in early G₁ cells in which poly(A) RNA was detectable. These data suggest that the clustering of snRNP antigens into discrete regions after mitosis occurs only after the appearance of poly(A) RNA, presumably following the establishment of transcription by RNA polymerase II (pol II).

Discussion

This work describes several previously unknown aspects of the functional organization of the nucleus. In cells ranging from sea urchin to human, nuclear poly(A) RNA was concentrated in several discrete transcript domains which often surrounded nucleoli. These areas appeared to be interiorly located, had defined borders, and corresponded to areas of very low DNA concentration. There seemed to be complete overlap in these regions between poly(A) RNA and snRNP antigens, providing the best evidence to date that these are sites of pre-mRNA processing. Placement of these areas with respect to total DNA, replicating DNA, and centromeric DNA strongly indicates that these putative processing areas are specifically positioned with respect to underlying chromatin, which may have profound implications for higher-level nuclear organization. Transcript domains were clearly separate from areas of late replicating DNA indicating a distinct physical partitioning of two separate nuclear functions during late S-phase. Finally, our data indicate that the clustering of snRNPs into discrete nuclear domains in early G₁ and the appearance of corresponding low density DNA regions occur only after the reappearance of poly(A) RNA. This raises the interesting possibility that nascent pol II transcripts play a significant organizational role in the formation of snRNP clusters, similar to the postulated role of pol I transcripts in the assembly and maintenance of nucleoli (reviewed in Scheer and Benevente, 1990).

The Relationship of Transcript Domains to pre-mRNA Processing

While snRNP antigens have been described by several laboratories to have various degrees of clustering (see Introduction), the subnuclear distribution of total poly(A) RNA, the major substrate for snRNP processing, has not been previously investigated. Our results support the functional significance of clustered snRNP protein distributions (Fakan et al., 1984; Nyman et al., 1986; Ringertz et al., 1986; Spector, 1990) and, moreover, provide the first evidence for these as active processing centers rather than assembly and storage sites. Since the completion of the work presented here, the validity and role in processing of previously reported clustered snRNP distributions has been called into question by Carmo-Fonseca et al. (1991a,b), who present results interpreted to indicate that essentially all of the extremely abundant snRNAs are localized together in just one to four small nuclear foci. The interpretation of these few foci as the nuclear processing centers is difficult to reconcile with our results which co-localize pre-mRNA and

Figure 7. Distribution of poly(A) RNA and snRNP antigens in mitotic and postmitotic HDFs. In the vast majority of interphase nuclei, the distributions of poly(A) RNA and snRNP were virtually identical and always similar to the example shown in Fig. 3, *C* and *D*. In experiments stained simultaneously for DNA, poly(A) RNA, and snRNPs—mitotic and early G₁ cells were readily identified based on their distinct morphology, DNA condensation, and distribution of poly(A) RNA and snRNPs. For example, while all other interphase cells had no detectable cytoplasmic snRNPs, distinct pairs of presumably early G₁ daughter cells had high levels of cytoplasmic snRNP signal, along with condensed nuclear DNA and little or no nuclear poly(A) RNA signal. Poly(A) RNA (*left*) and snRNP (*right*) are shown for cells at metaphase (*A*), telophase (*B*), very early G₁ (*C*), and early G₁ (*D*). Arrows in *D* indicate newly formed transcript domains and corresponding regions of snRNP clustering. DAPI staining of DNA (*blue*) is shown in *A* and *B*. DAPI was not photographed in *C* and *D* to demonstrate more clearly the distribution of poly(A) RNA and snRNPs in the nucleus.

snRNP antigens to 10–20 widely distributed domains of relatively equal intensity, or with the observation that the snRNA cap structures localize to snRNP antigen clusters (Reuter et al., 1984). The potential for these areas in RNA processing is also supported by recent studies showing that microinjected β -globin pre-mRNA co-localizes with snRNP clusters (Wang et al., 1991).

Our work also supports the validity of clustered snRNP patterns by defining a physical compartment in the nucleus which was observed using three separate approaches: (a) in situ hybridization to poly(A) RNA; (b) immunofluorescence to snRNP particles; and (c) visualization of total DNA with fluorescent dyes. This, along with the fact that these regions were apparent in unfixed cells stained for DNA, greatly increases the confidence with which it can be concluded that these are genuine structural and functional nuclear compartments. Our data are consistent with numerous EM studies indicating interior nuclear regions of low DNA density (reviewed in Fakan, 1978; Fakan and Puvion, 1980; Fawcett, 1981) in which snRNP antibodies localize (Fakan et al., 1984), but do not necessarily support the conclusion that these regions contain no DNA (Spector, 1990). It is extremely difficult to distinguish between the presence of no DNA and little DNA in these regions using EM or by advanced fluorescence imaging using DNA-specific dyes (Carter et al., manuscript in preparation), hence a conclusive answer to this question awaits more extensive analyses.

Does Transcription Occur in These Regions?

There are at least three reasonable models that would explain why the vast majority of nuclear poly(A) RNA resides in these defined transcript domains. First, this RNA might be transcribed elsewhere and be transported to these regions to be polyadenylated and spliced. Similarly, this RNA might be both transcribed and polyadenylated elsewhere and then transported to these sites for further processing which would be rate limiting. Alternatively, transcription, polyadenylation, and splicing may all occur within transcript domains, in which case any one of these processes could be rate limiting and cause a build-up of transcripts.

Several lines of evidence presented here are consistent with the possibility that poly(A) RNA regions are transcription sites. These include: (a) The distribution of poly(A) RNA is not random with respect to total DNA; (b) these discrete regions contain very little DNA, which one might expect in areas of active transcription since much evidence indicates that active chromatin is decondensed (Weisbrod, 1982; Lewin, 1990); (c) centromeric and late replicating DNA, which are transcriptionally inactive, are completely excluded from these regions; and (d) early-replicating DNA, which contains most active genes, was not preferentially excluded and seemed to partially overlap poly(A) RNA regions. It is tempting to visualize these regions as filled with loops of decondensed transcriptionally active DNA analogous to structures seen on amphibian oocyte lampbrush and drosophila polytene chromosomes. However, poly(A) transcript domains would reflect a significantly different level of organizational complexity; with each poly(A) RNA domain reflecting transcriptional activity from hundreds or thousands of individual genes on different chromosomes rather than the activity of a single atypically large or amplified transcription unit. However, short of localizing specific active

genes directly within these regions, it cannot be ruled out that there is nonspecific exclusion of DNA in this region imposed by the concentration of transcripts and splicing machinery.

The fact that snRNP antigens are located within transcript domains may provide indirect evidence that pol II transcription occurs at these sites: Transcription by pol II is necessary for polyadenylation and splicing in vivo (Smale and Tjian, 1985; Lopata et al., 1986; Sisodia et al., 1987) and EM studies have shown RNP particles and apparent splicing on nascent pol II transcripts (Beyer et al., 1981; Osheim et al., 1985; Beyer and Osheim, 1988) and snRNP antigens within spread chromatin (Fakan et al., 1986). Likewise, a functional polyadenylation signal is necessary for the termination of pol II transcription, indicating a tight linkage between 3' end processing and transcription (Falk-Pederson et al., 1985; Whitelaw and Proudfoot, 1986; Logan et al., 1987; Connelly and Manly, 1988), and at least two studies indicate that non-transcribed promoter region sequences can affect mRNA processing and transport (de la Pena and Zasloff, 1987; Neuberger and Williams, 1988). However, because both polyadenylation and splicing occur on pre-mRNAs added exogenously to nuclear extracts, and certain full-length primary transcripts are seen by Northern analysis, the possibility remains that the progression from transcription to polyadenylation and splicing takes place in a linked manner but that the physical compartmentalization, in some cases, may be separate.

Further Implications for Nuclear Structure and Function

If transcript domains do represent transcription sites, this would indicate that active chromatin is distributed in clusters throughout the interior portion of the nucleus. Direct and conclusive evidence for the general location of active genes at interphase is lacking. Several laboratories using EM autoradiography have observed nascent RNA at sites throughout the nuclear interior, in some cases proximal to structures of unknown function seen in EM micrographs (reviewed in Fakan and Puvion, 1980). In contrast, others have shown DNase I-sensitive DNA, presumed to represent transcriptionally active chromatin, primarily near the nuclear envelope in some cell types (Hutchinson and Weintraub, 1985; Kryostek and Puck, 1990). However, many studies indicate that the bulk of DNA, including centromeres and the inactive X chromosome, occurs in heterochromatic regions at the nuclear periphery (Fawcett, 1981; Ford, 1973). In this context an earlier study noted that poly(A) RNA was distributed throughout the nucleus and not just at the periphery, although subnuclear details were not investigated (Bauman et al., 1990).

Consistent with a more interior positioning of active chromatin, our previous work directly localized the Epstein-Barr Virus genome to the inner 50% of the nuclear volume in lymphoma cells where it is abundantly transcribed (Lawrence et al., 1988, 1989). Similar positioning was seen for the Human Immunodeficiency Virus (HIV) genome in productively infected cells (Lawrence et al., 1990) and preliminary studies suggest an interior position for some endogenous active pol II genes (Xing, Johnson, and Lawrence, manuscript in preparation). Tracks of EBV nuclear RNA extend from the internally localized gene into the outer 50%

of nuclear volume, often appearing to contact the edge of the nucleus (Lawrence et al., 1989) and are completely preserved both spatially and quantitatively during nuclear matrix preparations (Xing and Lawrence, 1991). Thus, while internal localization of poly(A) RNA in transcript domains is difficult to reconcile with often cited views of nuclear organization which propose localization of active chromatin at the nuclear periphery, our data are not necessarily inconsistent with proposals that the nuclear lamina or pores might function in nuclear organization (for example, Blobel, 1985). The potential compartmentalization of various functions seen here and in previous work from our lab and others' concerning the association of RNA (Xing and Lawrence, 1990; reviewed in Fey et al., 1991) and replicating DNA (Nakayasu and Berezney, 1989; reviewed in Nelson et al., 1986) with the nuclear matrix make it reasonable to suggest that the nuclear matrix may play a role in this physical partitioning of various nuclear functions.

The observation that poly(A) RNA is often preferentially concentrated around the nucleolus raises questions as to whether this structure or the region immediately surrounding it plays a role in mRNA transport and/or processing. Interestingly the HIV rev protein, a predominantly nucleolar protein, is involved in transport of HIV mRNAs from the nucleus (Cullen et al., 1988; Cochrane et al., 1989; Lawrence et al., 1991). It is possible therefore that a direct association exists between mRNA transport and nucleolar function. For example, it is conceivable that ribosomal subunits or other nucleolar constituents are physically associated with mRNAs during transport.

In conclusion, we have provided evidence for functionally significant physical partitioning of RNA and DNA within the nucleus, and have described a compartment which is likely to play a major role in pre-mRNA processing and, possibly, transcription. Furthermore, the relative ease with which this major intra-nuclear compartment can be visualized by any of the techniques used, will provide a landmark for studying the precise relative placement of other important nuclear constituents such as active genes.

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