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Connecting Nuclear Architecture and Genomic Function

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Research in our laboratory employs a dual approach to correlating genomic function and regulation with nuclear architecture. In one series of projects we are identifying, cloning, and studying the molecular, genetic, and functional properties of the nuclear matrix proteins which comprise the three-dimensional nuclear architecture. In a second series, we are using molecular labeling, microscopic, and computer imaging approaches to visualize in three dimensions sites of genomic organization and function within the in situ nuclear architecture and to obtain precise structural and spatio-temporal information about the individual sites where genomic function and regulation occurs. Additionally, our group is investigating the possible arrangement of the functional sites into higher-order domains and their relationship to the overall architecture and regulation in the cell nucleus.

Nuclear Matrix Proteins as a Basis for Genomic Organization, Function, and Regulation

While a plethora of functional properties are associated with the isolated nuclear matrix [Berezney, 1991; Berezney et al., 1995], our knowledge of the proteins which compose the nuclear matrix is still in its infancy. Comprehensive analysis of the individual proteins is, therefore, critical as a basis for correlating higher order structural organization in the nucleus with the associated genomic functions.

Studies in our laboratory are emphasizing the major proteins of the nuclear matrix (termed nuclear matrins) which are common among mammalian cells [Nakayasu and Berezney, 1991; Belgrader et al., 1991a]. Microsequencing, molecular cloning and immunoblot analysis has suggested that a significant portion of the major nuclear matrix proteins correspond to known pre-mRNP proteins, transcriptional or RNA splicing factors [Mattern et al., 1996; S. Kim, X. Wei, and R. Berezney, unpublished findings]. Additional proteins are involved in other regulatory properties or are newly identified proteins whose function remains to be resolved. Our research group is currently concentrating its efforts on three nuclear matrix-associated proteins termed matrin 3, matrin cyp, and matrin p250.

Matrin 3 is an acidic nuclear matrix protein (125 kDa). Antibodies to this protein decorate the nuclear interior with a fibrogranular pattern typical of the nuclear matrins [Nakayasu and Berezney, 1991; Belgrader et al., 1991b]. The analysis of matrin 3 human and rat cDNA sequences indicates a high degree of conservation among mammals [Belgrader et al., 1991b]. The lack of homologies with other proteins or functional motifs gives few clues to possible function, although the presence of over 40 potential phosphorylation sites suggests the role of protein phosphorylation. As a prelude to studying matrin 3 function, we are investigating the genomic organization of the matrin 3 gene in the rat. Preliminary results suggest the presence of at least two closely related genes and the possibility of alternatively spliced transcripts [Mortillaro et al., 1993; Somanathan et al., 1995].

Matrin cyp is a 106 kDa protein that is localized in the nucleus and highly enriched in the nuclear matrix [Mortillaro and Berezney, 1995]. Analysis of the predicted amino acid coding sequence revealed a 170 amino acid stretch at the N-terminal region which showed high identity with cyclophilins, a family of ubiquitous proteins that bind to the immunosupressant drug, cyclosporin A, and catalyze the conversion of cis-proline to trans-proline [peptidyl-prolyl-isomerase (PPIase) activity]—an event impor-

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tant for protein folding [Fisher et al., 1989; Gething and Sambrook, 1992]. A fusion protein containing the cyclophilin domain of matrin cyp has PPIase activity that is completely abolished by cyclosporin A [Mortillaro and Berezney, 1995]. The matrin cyp coding region also contains an acidic serine-rich region similar to the nuclear localization signal-binding (NLS-binding) domains described for Nopp 140 by Meir and Blobel [1992], and a series of serine-arginine (SR) repeats dispersed throughout the C-terminal half of the protein. SR repeats are characteristic of splicing factors [Zahler et al., 1993] and have been demonstrated to target proteins to splicing factor-rich, nuclear speckles [Li and Bingham, 1991]. The presence of SR repeats in matrin cyp *is consistent* with the localization of matrin cyp at splicing factor-rich nuclear speckles in both whole cells and following extraction for nuclear matrix [Berezney et al., 1995].

The observations that matrin cyp contains a functional cyclophilin domain, a putative NLS-binding domain, and is present in both splicing factor-rich nuclear speckles and the cytoplasm (in low levels) leads us to speculate that matrin cyp may associate, via its cyclophilin and NLS-binding domains, with newly synthesized nuclear protein in the cytoplasm, and by acting as a molecular chaperone, recruit these proteins to the nuclear speckles. Alternatively, matrin cyp may function solely as an intranuclear targeting protein.

Matrin p250, an approximately 250-kDa protein of the nuclear matrix, was recently demonstrated to be identical to a hyperphosphorylated form of RNA polymerase II large subunit [Mortillaro et al., 1996]. To our initial surprise, significant portions of p250 localized at splicing factor-rich sites in both intact cells and cells extracted from the nuclear matrix. The significance of this observation was reinforced by experiments which demonstrated the association of p250 [hyperphosphorylated but not hypophosphorylated forms of pol II-LS] with pre-mRNA splicing complexes assembled in vitro and with a subset of snRNP and SR protein splicing factors [Mortillaro et al., 1996]. These results implicate nuclear matrix-associated, hyperphosphorylated pol II-LS as a putative linkage factor for coordinating transcription and RNA splicing processes in the cell nucleus.

Spatio-Temporal Analysis of DNA Replication Sites Using Three Dimensional Microscopy and Computer Image Analysis

Fluorescence microscopic analysis of newly replicated DNA has revealed discrete granular sites of replication (RS). The average size and number of RS from early- to mid-S-phase suggests that each RS contains numerous replicons clustered together [Nakayasu and Berezney, 1989; Berezney, 1991; Berezney et al., 1995]. We are using fluorescence laser scanning confocal microscopy (LSCM) in conjunction with multi-dimensional image analysis (MDA) to gain more precise information about RS and their spatio-temporal relationships. Individual RS are optimally visualized following short pulses with BrdU in mouse 3T3 fibroblast cells. Using newly improved imaging techniques, we can discriminate over 1×10^3 RS following a 2 min pulse of cells synchronized in early S phase with each site having an average x or y plane length of about 0.4 microns.

Double labeling experiments performed at two different times (pulse-chase-pulse) enabled us to examine the relationship of early-versus later-replicated DNA at individual sites. Cells in early S phase were labeled for 2 min with CldU (FITC secondary antibody), chased for different times, and pulsed again for 5 min with IdU (Texas Red secondary antibody). As a control, simultaneous pulsing with both CldU and IdU resulted in the virtually complete overlap of all the replication sites with the two probes (yellow sites). Following a 15-min chase, over 50% of the total RS were co-localized and decorated granular RS similar to those observed after a single 2 min pulse. Later pulsed replication sites (red sites) were consistently observed in juxtaposition to early ones (green or yellow sites). Increasing the chase time between pulses resulted in increased distances between early (green) and later (red) RS, as well as increased separation among early and later labeled sites until they were completely separated after a one-hr chase.

These results lead us to consider the existence of higher-order spatial domains of neighboring RS whose replication may be temporally regulated. To investigate this further, we performed long-term double labeling (pulse-chase-pulse) experiments. 3T3 cells in early S-phase were pulsed for 1–5 hr with CldU, chased for 0–4 hr and pulsed a second time for 3 hr with IdU. Initial

results suggest that a significant portion of the temporally distinct RS occupy separate spatial domains within the nucleus. Arrays of RS in close proximity were often observed within these domains.

We are currently applying computer-aided pattern recognition techniques to elucidate the 3-D higher-order assembly of individual replication sites. Using a "nearest neighbor" center to center distance of 0.6 microns, we find that the approximately 1,000 sites with average diameters of 0.4 microns detected after a 2 min pulse separate into approximately 50 discrete higher-order "replication domains" (average distance between sites of ≤ 0.2 microns), or about 20 replication sites per domain.

In another series of experiments, we are following the fates of individual replication sites throughout the cell cycle of 3T3 fibroblasts. Previous studies from our laboratory and others [Meng and Berezney, 1991; Spavoli et al., 1994; Berezney et al., 1995], revealed that the arrangement of replicated DNA into "RS-like" structures persists throughout the cell cycle and subsequent daughter cells. These results may be a consequence of the three-dimensional arrangement of chromatin into precise domains of clustered loops. We have designed "double pulse-double chase" experiments to determine whether the DNA sequences replicated at individual RS are precisely maintained as the cell progresses through the cell cycle. RS were labeled in early S (green probe) and two hours later (red probe). Our results demonstrate the maintenance of the temporally distinct replicated DNA into spatially distinct sites (separate green- and red-labeled sites) throughout the 8 hr S-phase. Moreover, the separate green and red replication sites were predominantly maintained following cell division and in subsequent cell generations. In future experiments we will determine the specific genomic sequences (genes) at individual RS and the cell cycle dynamics of these associations.

In summary, our combined LSCM-MDA results indicate that DNA replication occurs at approximately 1,000 distinct sites at any given time in early to middle S-phase. "Pulse-chase-pulse" double labeling experiments reveal that the average RS takes about 1 hr to complete replication. Assuming approximately 5 times 10^4 replicons per nucleus and an average S-phase of 8 hr, each RS would contain an average of six replicons which are replicated in a relatively synchronous wave. The labeled DNA remains organized in "replication-like sites" throughout the cell cycle and subsequent cell generations. This suggests that the imaged sites are not only sites of replication but are a fundamental aspect of the higher order structure of the genome and its organization inside the cell nucleus. An even higher level of organization was indicated by pattern recognition image analysis which revealed that the individual RS are three-dimensionally arranged into a series of higher order domains (approximately 20 RS per domain). Experiments are in progress to investigate the gene sequences being replicated at individual RS by combining in situ hybridization with our high precision LSCM-MDA techniques. The possible relationship of higher order replication domains to individual chromosome territories is also under analysis. Finally, we are currently applying these visualization and computer imaging approaches to sites of transcription and RNA splicing factor-rich sites in the cell nucleus.

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