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Connecting Genomic Architecture and DNA Replication in Three Dimensions

Ronald Berezney, Hong Ma, Chungling Meng, Jagath Samarabandu and Ping-chin Cheng
Departments of Biological Sciences and Electrical and Computer Engineering, State University of New York at Buffalo, Buffalo, NY, 14260, USA

The cell nucleus is the repository for the genetic information of all eucaryotic cells including that of man. Despite enormous progress in defining molecular properties of the genomic functions of DNA replication, transcription and RNA splicing and processing, our knowledge of how these processes are organized and regulated inside the three dimensional structure of the cell nucleus is still in its infancy.

Research in our laboratory employ 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 compose the three dimensional nuclear architecture. A summary of our recent progress in this area is summarized in a separate abstract in this volume (see Mortillaro et al.).

The second direction of our research uses mulecular 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. Our present studies are concentrating on DNA replication and are summarized below.

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. 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 (2 min) in mouse 3T3 fibroblast cells. Using newly improved imaging techniques, we can discriminate over $1x10^3$ RS following a 2 min pulse of cells synchronized in early S phase (Figs. 1 and 2). Preliminary data suggests that the average number of detected sites following a 2

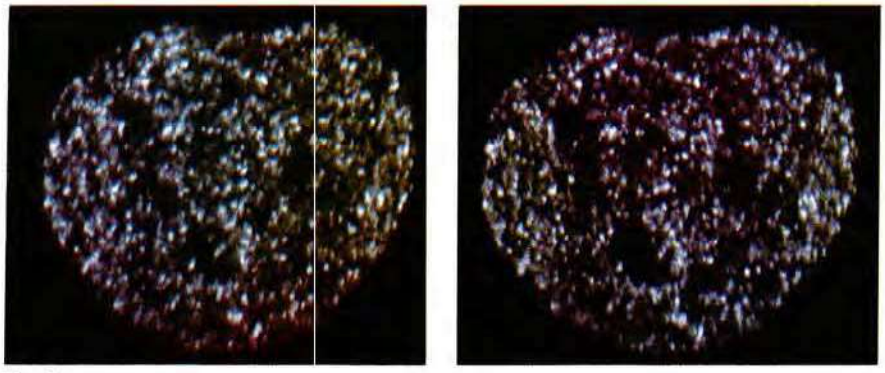

Fig. 1.

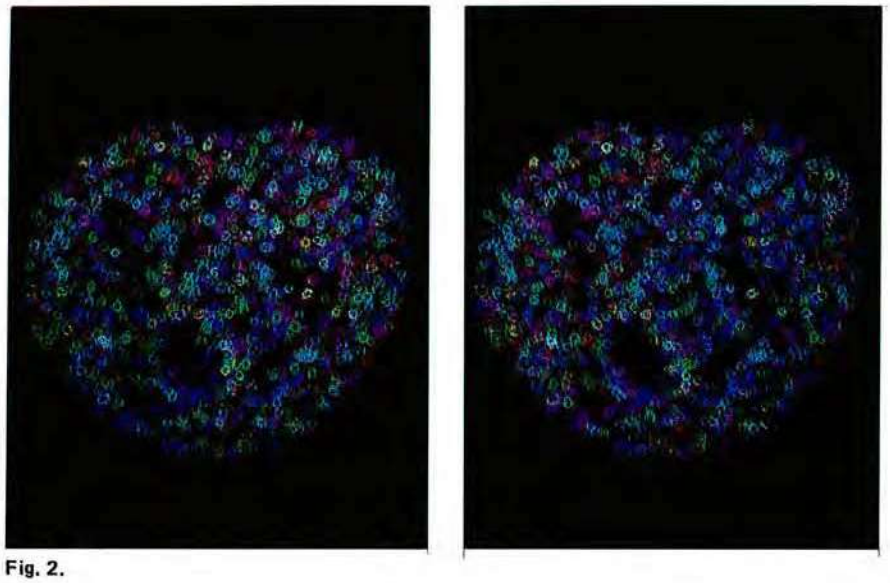

Fig. 2.

min pulse is approximately 600 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 virtual complete overlap of all the replication sites with the two probes (yellow replication sites, Fig. 3). 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 (Fig. 4). Later pulsed replication sites (red sites) were consistently observed in juxtaposition to early ones (green or yellow sites). Increasing the chase time between pulses to 1-2 hr resulted in an increasing spatial separation between early and later Rs (Fig. 5).

These results led 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 pattern recongnition 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 600 sites with average diameters of 0.4 microns detected after a 2 min pulse, separate into approximately 60 discrete domains (average distance between sites of < 0.2 microns) or about 10 replication sites per domain. The average number of domains per nucleus is reduced to about 30 with a center to center distance of 0.9 microns (< 0.5 micron distance between sites).

In another series of experiments we are following the fate of individual replication sites throughout the cell cycle of 3T3 fibroblasts. Previous studies from our laboratory 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 chromation into precise domains of clustered loops. We have designed "double pulse - double

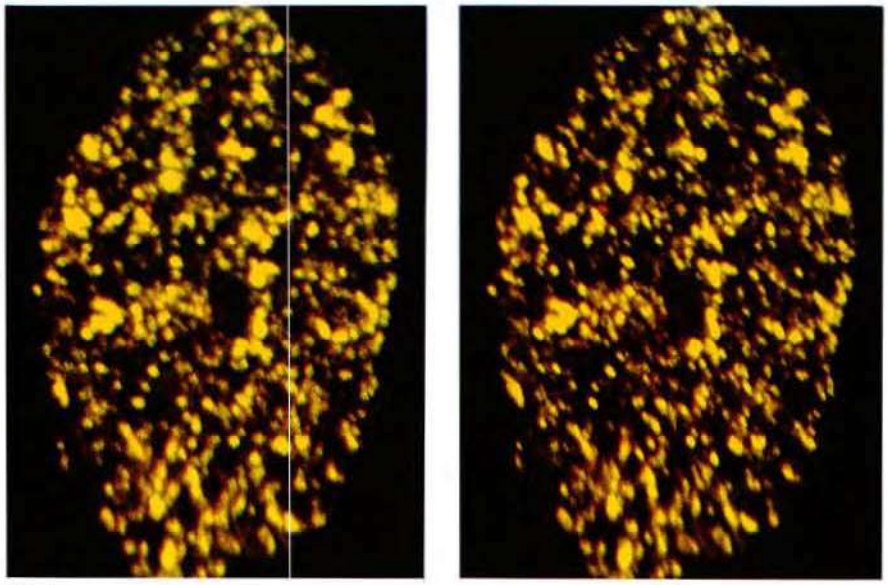

Fig. 3.

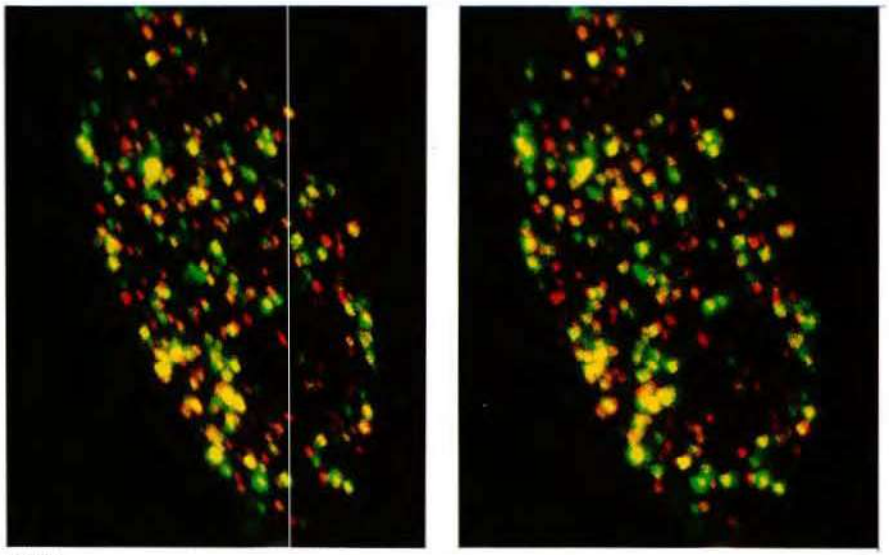

Fig. 4.

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. The G_2-plase, however, was characterized by a significant mixing of DNA between the two populations of sites (~30% yellow sites). The percent of "DNA mixing" between the two populations of sites increased to >50% 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

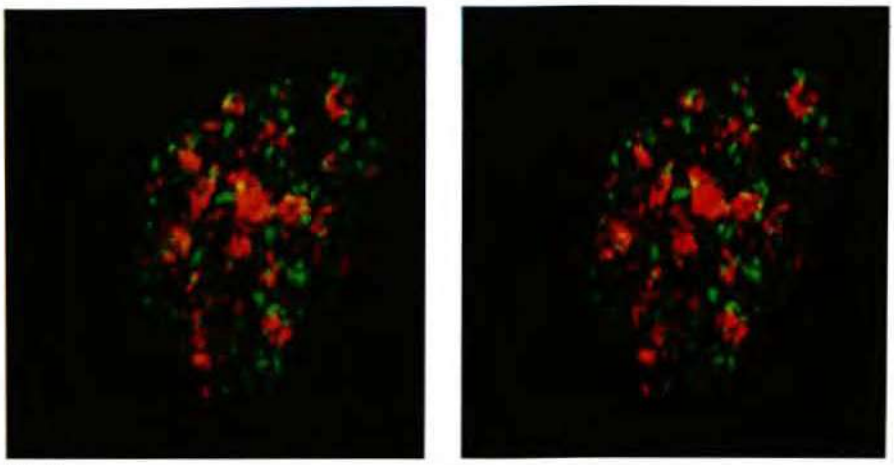

Fig. 5.

indicate that DNA replication occurs on an average of 600 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 4-8x10^4 replicons per nucleus, this suggests that each RS contains an average of 5-10 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 10 RS per domain). (Supported by NIH grant GM-23922)