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Temporal and Functional Analysis of DNA Replicated in Early S Phase

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Background and Previous Studies

Synchronously proliferating cells, both in vivo and in culture, are most vulnerable to tumor-inducing effects of short-lived chemical carcinogens when the cells are treated in the S phase of the cell cycle (Chernozemski *et al.*, 1970; Craddock, 1973; Bertram and Heidelberger, 1974; Craddock and Frei, 1974; Pound and Lawson, 1975) and more particularly at the beginning of the S phase (Grisham *et al.*, 1980; Kaufmann *et al.*, 1981; Kaufmann *et al.*, 1987a; Kaufmann *et al.*, 1987b; Kaufmann and Kaufman, 1993; Kaufman *et al.*, 2007). Furthermore, other studies have shown that carcinogen binding to DNA is greatly increased at replication sites (Cordeiro-Stone *et al.*, 1982; Paules *et al.*, 1988). Based on these two findings we reasoned that genomic sites replicated early in S phase include important target sites for carcinogenesis (Grisham *et al.*, 1983). To test whether this hypothesis is true we have undertaken a program of studies that involved the mapping of early replicating genomic regions, including the identification of genes present in these regions, and thereafter we have investigated the program of DNA replication through some of these earliest replicating domains. While these studies were done with the hope they would be informative about the mechanism for the vulnerability of cells to transformation at the start of the S phase, they have also been revealing about the organization of DNA replication particularly during the early S phase. In this case, as with many other areas of biological investigation, the more carefully one looks at DNA replication, the more complex, more intricate and dazzling the process turns out to be.

Efforts to identify genomic targets underlying the exceptional vulnerability early in the S phase were predicated on the belief that there would be orderliness to replication during this interval. This seemed likely since it had been shown that the same genomic regions replicated in approximately the same time in successive S phases and some genes or genomic regions could be categorized as replicating in the first or second half of the S phase. Progress toward this goal of further characterizing the orderliness of replication was greatly enhanced by the technique for synchronization of cell proliferation using aphidicolin which allowed hourly intervals through the S phase to be resolved (Cordeiro-Stone and Kaufman, 1985). With this synchronization method the timing of replication of specific genes could be assigned to specific hourly intervals through the S phase (Doggett *et al.*, 1988). These and other studies showed that replication was highly ordered and the order was very

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reproducible (Iqbal *et al.*, 1987; Sorscher *et al.*, 1993; Jackson and Pombo, 1998; Norio *et al.*, 2005; Desprat *et al.*, 2009).

It was shown that the synchronization protocol did not stop replication completely but allowed it to begin and proceed in the normal order but at a greatly reduced rate (Sorscher and Cordeiro-Stone, 1991). Because of this property we were able to perform studies where BrdU was added to the medium of cultured cells prior to the start of DNA replication and replication was allowed to proceed through a short interval of the S phase. This experimental strategy permitted us to do a study where the earliest replicated regions in S phase were labeled with BrdU while in the presence of aphidicolin. Thereafter the cells were washed free of aphidicolin and collected when they reached metaphase. The mitotic figures were analyzed for the chromosomal band location of BrdU labeling (Cohen *et al.*, 1998). For each chromosome, the locations of labeling were assigned to chromosomal bands. Since cells exposed to this aphidicolin regimen enter the S phase at slightly different times, DNA replication had proceeded to different extents, with late entering cells less labeled. Consequently these cells yielded mitotic chromosomes with fewer or more labeled bands per chromosome. So for each chromosome the number of bands that were labeled was determined and chromosomal labeling was stratified according to the number of labeled bands (one, two, or three, etc. labeled bands). For each chromosome very few sites were labeled consistently in early S phase even when there were several labeled bands per chromosome. As the analysis of early replicating bands progressed to chromosomes with fewer labeled bands it became evident that one band (two in the case of chromosome 15) was consistently labeled before the other bands (i.e., it had a high labeling frequency particularly when there were no other labeled bands). In this manner it was possible to determine that there were only a few chromosomal bands (the six most prominent sites were in five chromosomes: 1p36, 8q24, 12q13, 15q22, 15q15, 22q13) that displayed this pattern of labeling with high frequency and therefore corresponded to labeling at the earliest time in S phase. Consistent with this interpretation, the frequency of labeling in these bands decreased as the numbers of labeled bands in a chromosome increased later in S phase. Thus, the S phase appears to begin at relatively few genomic sites, and these sites ceased to be labeled later in S.

Using the same synchronization and BrdU labeling technique, we labeled DNA synthesized as the bulk of cells entered S phase and recovered the density labeled DNA in CsCl gradients. This DNA was used to generate a library of about 10,000 clones, then the cloned DNA was sequenced and the clones mapped to the human genome (Brylawski *et al.*, 2000). In analyzing the genomic mapping of the clones, attention was focused on genomic sites where clusters of overlapping clones defined contig regions of at least 100 kb, i.e., where three or more 40 kb clones partially or completely overlapped (Cohen *et al.*, 2006). Isolated single clones were omitted as possible contaminants. Analysis of the numerous contigs of clustered clones showed that they were enriched in sequence elements previously reported to be associated with early replicating DNA (Woodfine *et al.*, 2004). Of note, each of the chromosomal bands that were previously found to be prominently labeled early in S phase (Cohen *et al.*, 1998) had one or more of the clusters mapped to them (Cohen *et al.*, 2006). Genes mapped within the contigs were analyzed for gene ontology to determine whether any gene families were overrepresented in this early replicating fraction. This analysis showed that the genes in the contigs had an over-representation of genes in the apoptosis and Wnt signaling pathways, as well as an abundance of genes in the base-excision DNA repair family (Cohen *et al.*, 2006; Cohen *et al.*, 2007). Similar analysis of data from Woodfine and collaborators (Woodfine *et al.*, 2004) where the early replicating regions were identified using hybridization to tiled genomic DNA microarrays with human lymphoblastoid cell DNA, also found overrepresentation of the apoptosis genes. These findings (Cohen *et al.*, 2006) were particularly notable because alterations of the function of genes in these three

families are found often in human cancers, particularly abnormalities of several genes in the apoptosis family. Mutations that suppress apoptosis in abnormal cells, or suppress the cellular differentiation program mediated by Wnt family genes, can easily be imagined to lead to the persistent propagation of abnormal cells or of cells that fail to terminally differentiate. If they also fail to respond optimally to DNA damage, compromising the efficient repair of DNA damage, such changes might contribute to the greater propensity of cells treated early in S phase to persist, accumulating DNA damage, and become genetically unstable. Such cells have increased risk of progressing to malignancy.

Studies of DNA and Chromatin Fibers

In a parallel track our research has been exploring the features of DNA replication early during the S phase to determine whether there are unique characteristics of replication in this interval that might contribute to its vulnerability to those carcinogen-induced effects that heighten the risk of cancer development. Our recent studies to expand our understanding of DNA replication early in the S phase have used the techniques of DNA analysis on extended single DNA fibers (Bensimon *et al.*, 1994; Bensimon *et al.*, 1995; Jackson and Pombo, 1998; Merrick *et al.*, 2004). Recently, similar approaches have been used to extend chromatin fibers (Cohen *et al.*, 2009; Cohen *et al.*, 2010). Extended chromatin fibers from animal cells have been used to show the distribution of covalently modified histones in some silent chromatin sequences (Sims *et al.*, 2006) and the organization of associated histones and DNA replication timing in centromeric regions (Blower *et al.*, 2002; Sullivan and Karpen, 2004; Lam *et al.*, 2006). In addition, chromatin fibers from plant cells have been utilized to study DNA replication (Quelo and Verbelen, 2004) and for high-resolution fluorescence in situ hybridization studies (Lavania *et al.*, 2003). Our studies involve the lysis of cells on a slide and the migration of DNA or chromatin fibers under the hydrodynamic pressure of a moving fluid meniscus that straightens the fibers. Different lysis conditions yield bare DNA fibers or intact chromatin fibers. DNA precursors incorporated into DNA and chromatin-associated proteins are detected by fluorescently labeled antibodies. In some cases labeled probes for defined genomic sites are also employed to identify specific regions of DNA on the extended fibers by Fluorescent In Situ Hybridization (FISH). With the sequential incorporation of distinguishable DNA precursors this approach allows the direction of DNA replication to be determined and the locations of origins of DNA replication to be identified. When asymmetric FISH probes are used the orientation of the region is definable.

Analysis of DNA Replication Early in the S Phase

By treating cells sequentially with two or more DNA precursors it is possible to determine the direction of DNA replication on single DNA fibers. In a typical experiment cells are treated with IdU detected with a red fluorescent antibody stain followed by CldU detected with a green fluorescent antibody stain. Evaluation of DNA replication tracts resulting from such sequential labeling reveals multiple labeling patterns: fibers with only red or green labeling, fibers with red labeling flanked by connected green labeling, or green labeling flanked by connected red labeling, or fibers with a red track next to a connected green track (Figure 1). In some cases, extended regions with red alternating with green labeling are found that presumably are closely spaced repeats of the individual labeling patterns. These patterns of labeling can be interpreted as having arisen by particular stages of DNA replication. For example, fibers with red labeling flanked by green labeling are only explained by DNA replication being activated during the first labeling interval at an origin within the central red-labeled track with bidirectional replication continuing in the two flanking green-labeled tracks. Similarly green-labeled tracks flanked by red-labeled tracks can only be explained by replication where two regions of replication converge at a

termination site in the green labeled region during the period of CldU Regions labeling. where multiple green and red labeled regions alternate must represent clusters of replicons that are contemporaneously activated along a DNA fiber, and with origins that are sufficiently closely spaced so that DNA replication in these units can be completed during the interval of labeling. Most of the labeled tracks that are detected have red labeling with adjacent green labeling present on only one side of the red track. This pattern of labeling, which we refer to as “extensions”, largely represent sites of progression of DNA synthesis that are not near origins or termini of bidirectional replication. It is possible that the same labeling pattern would be found at sites where DNA replication is activated at an origin and progresses unidirectionally, and at sites of termination of replication, where replication activated in adjacent replicons do not converge to a terminus simultaneously.

We have applied the DNA fiber labeling technique to analyze the properties of DNA replication during the first hour of synchronized DNA replication and compared this data to those of DNA replication during the fourth hour of the S phase (Frum *et al.*, 2008). Initially, we used aphidicolin to synchronize DNA replication and performed sequential labeling of DNA replication using variable intervals of IdU labeling (10, 20, and 45 min) followed by 20 min of labeling with CldU (green). At the end of the labeling interval, cells were recovered by trypsinization, washed extensively and then carefully quantified numbers of cells were applied onto siliconized glass slides and allowed to air-dry until the fluid volume was greatly reduced (Figure 1). Lysis solution was applied to the cells and after 10 min slides were adjusted to a 15 degree incline from vertical and the fluid allowed to run off the slide. Next the slides were air-dried, fixed with methanol-acetic acid, treated with HCl and washed extensively with buffer. Slides were treated with bovine serum albumin to block non-specific binding of antibodies. Thereafter a three level antibody amplification was used to detect both the IdU and CldU label incorporated into cellular DNA. Stained slides were coded and randomized prior to observation with a confocal laser scanning microscope. Evaluation of coded slides blinded the observer to their source (i.e., whether they were control or experimental samples) while they photographed labeled DNA fibers. For labeled fibers in the photographic images, red and green labeled tracks were distinguished and the lengths of the red and green segments were measured. The coding of the slides was broken only after all slides were scored. Thereafter the results of the experiments were compiled. We expected to find that the lengths of the red tracks would increase linearly with the length of the IdU labeling period. Instead, we found that for replication that initiated at origins (green-red-green tracks) activated in the first hour of S phase the lengths of the red tracks did not increase between those pulsed for 10 min and those pulsed for 20 min with IdU (Figure 2). In other words, there was pausing of replication near origins following their activation in the early S phase. We did not observe this pausing at sites remote from origins (red-green tracks) or at termination sites (red-green-red tracks). We also did not observe pausing in any of the samples from cells labeled during the fourth hour of the S phase. Thus, pausing did not occur at sites distant from origins or at later times in S phase. When we assessed pausing in malignant T98G (glioblastoma) cells it was absent even at origins activated in the first hour of the S phase. Further studies showed pausing to be absent in HeLa cells as well. Recognizing that pausing resembled the inhibition of chain elongation observed when DNA replication checkpoints are active, we treated the normal NHF1 cells with caffeine, an inhibitor of the intra-S checkpoint, and found that it could ablate pausing in normal cells. Since we were concerned that pausing might be an artifact of aphidicolin synchronization, we repeated these experiments but used release from confluence arrest or from serum starvation to synchronize the normal NHF1 cells and T98G cells, respectively. We found that the same results were obtained as when we used aphidicolin for synchronization. Thus, pausing is a feature of replication from origins activated in the first hour of the S phase in normal cells and not an artifact of the synchronization procedure (Frum *et al.*, 2008).

Using the two precursor labeling process to distinguish different fluorescent labeling patterns of replication as single origins, elongations, clusters and terminations, we quantified the prevalence of each of these patterns at each hour of the S phase for both normal and cancer cells (Figure 3) (Frum *et al.*, 2009). In addition, we quantified the lengths of the labeled tracks in each case. Conditions for the synchronization of the two cell lines yielded similar rates of progression through the S phase. As might be expected, we found that single origins were activated most frequently during the first hour of S phase for both the normal and cancer cells. There was a more rapid reduction in activation of single origins in subsequent hours in the normal NHF1 cells, which suggests that coordination of origin firing in the cancer cells is abnormally regulated in the first hour of S phase. Again, as might be expected, terminations were most abundant in the latter half of S phase for both the normal and cancer cells, but terminations began to increase in frequency earlier (5th hour) in normal cells and peaked in the 6th hour whereas the peak for the cancer cells was in the 7th hour, again suggesting an alteration of regulation of terminations in the cancer cells. At all times in the S phase, structures that we designated as unidirectional elongations were the most abundant type found for both cell types, representing 50% to 80% of the scored fibers. Minor differences between the frequencies of elongations in normal and cancer cells could be explained by the greater abundance of clusters of labeled replicons in the middle of S phase and the earlier onset of terminations seen in the normal cells. We measured the lengths of all of the labeled tracks and found with rare exceptions that the tracks in the normal cells were longer than in the cancer cells (Figure 4). The most notable exception was in the length of the tracks of the abundant single bidirectional origins that are activated during the first hour of S phase. Presumably the shorter length of these tracks in normal cells results from the pausing of replication near origins in normal cells during that time interval in the S phase (Frum *et al.*, 2009). Given that the two cell types complete replication in the same time interval based on cell cycle progression analysis, and because the lengths of labeled tracks in the cancer cells are usually shorter, we deduce that there must be a greater number of origins of replication per unit length of DNA that are activated in the S phase of the cancer cells. This implies that origins not active in the normal cells must be activated in the cancer cells to complete replication in the same time interval as the normal cells. It is possible that is slower in cancer cells and this is sensed as a form of replicative stress causing more origins to be initiated (Ge *et al.*, 2007; Ibarra *et al.*, 2008). It should be noted that we used a very restrictive definition of clusters of contemporaneously replicating replicons in these studies. This may explain the lower abundance of clusters that we found versus the results of studies previously published by others (Jackson and Pombo, 1998).

Analysis of DNA Repair on DNA Fibers

Because the DNA fiber analysis technique was able to detect sites of initiation of replication as well as sites where DNA was being elongated at active sites of replication, we employed this method to evaluate the inhibitory effects of UV-induced DNA damage on these processes in normal cells (Chastain *et al.*, 2006a). Groups of normal cells were treated by exposure to graded doses of UV light between intervals of DNA replication with IdU and with CldU. In agreement with observations using molecular analysis the studies with DNA fiber technology showed that both origin activation and elongation of newly replicated DNA at active replication sites could be inhibited by UV treatment at 1 or 10 J/m². Inhibition of replicon initiation at origins was inhibited maximally at the low dose of UV while inhibition of elongation of strands at sites of active replication showed a partial dose response to UV radiation. By the DNA fiber analysis technique these effects could be observed on single molecules rather than as the averages of thousands or millions of strands of DNA as are required using techniques of molecular biology.

An additional dimension to studies of DNA damage and repair were made possible by adding a means of detecting DNA damage sites, either sites of adduction or physical alteration of DNA structure, using fluorescently tagged reagents. Reactive oxygen species, whether generated endogenously in cells or resulting from exogenous exposures, react with and modify cellular macromolecules including several changes in DNA. When hydrogen peroxide (H_2O_2) is the reactive oxygen species, among the DNA lesions that develop are apurinic/apyrimidinic (AP) sites that form when the reaction of H_2O_2 with DNA causes bases to be lost from the sugar-phosphate DNA backbone. The AP sites that form can be detected with a biotin-tagged aldehyde reactive probe (ARP), which reacts with the ring-open form of abasic sites resulting in a biotin tag at the AP site which can be detected using fluorescence microscopy (Figure 5). Using these conditions, we were able to detect and quantitate AP sites in cells cultured under normal conditions or following stress with exogenous H_2O_2 . We detected DNA replication using the sequential incorporation of IdU and CldU. Exposures to H_2O_2 occurred after the IdU labeling and before the CldU labeling. There were background levels of AP sites observed even in controls presumably as the result of spontaneous events or due to H_2O_2 generated during signaling or as the result of metabolic reactions. Exposure to exogenous H_2O_2 significantly increased the frequency of AP sites per unit length of DNA (Figure 6) (Chastain *et al.*, 2010). The measured levels of AP sites in DNA from normal and H_2O_2 exposed cells closely agreed with the levels measured by the slot blot assay that is the “gold-standard” for quantitation of AP sites in DNA samples (Nakamura *et al.*, 1998; Nakamura and Swenberg, 1999). Besides a high level of accuracy in quantitating AP sites, the DNA fiber assay achieved a higher level of precision than did the slot blot assay. Measurements of the numbers of AP site per unit length in areas undergoing replication were made for cells cultured in standard medium versus those treated with medium containing exogenous H_2O_2 . We found that in cells exposed to 20 μM H_2O_2 , AP sites in newly replicated DNA nearly tripled in comparison to untreated controls (Chastain *et al.*, 2010). These findings show that there is increased susceptibility to oxidative damage in DNA regions undergoing replication. We demonstrated previously (Chastain *et al.*, 2006b) that there were clusters of AP sites present, indicating a non-random process of formation of the damage. It is plausible that the previously observed clustering of AP sites may have been due to the higher density of AP sites that form at replication sites. Relevant to the issue of clustering of DNA damage is whether DNA repair may be less efficient in locations where there are clusters of damage sites. It is at least plausible that efforts to repair damage at one AP site might inhibit the capacity for repair at a nearby site of damage. It should now be possible to determine whether this is correct or the opposite is true using the experimental approach described here.

Studies of DNA damage and repair could be even further refined by adding fluorescently labeled hybridization probes that can identify specific locations in the genome. In this manner it would be possible to monitor DNA damage and DNA repair at specific genomic loci. Application of this approach to studies of DNA damage and repair would be valuable because of the observation that AP site formation is not randomly dispersed. It is plausible that there may be particular genomic locations that are more susceptible to damage because of their location within the three dimensional structure of chromatin within the nucleus. It is recognized that there is a relationship between chromosomal location within the nucleus and transcriptional activity. Furthermore, it is recognized that the pattern of distribution of regions of DNA replication in the nucleus changes with time during the S phase. Thus, there may be a relationship between the potential for damage formation at particular sites and the time the sites are replicated during the S phase.

Genomic and Temporal Mapping of DNA Replication Origins Activated Early in S Phase

These concerns have prompted us to investigate replication dynamics associated with one of the early replicating regions we identified previously. Investigating such regions would permit us to demonstrate vividly the process of DNA pausing that occurs near origins that are activated very early in S phase and to determine whether these regions have an increased propensity for DNA damage occurring early in S phase. In earlier studies we used an assay that quantifies the abundance of nascent strands to identify origins and identified two adjacent origins related to a clone from our library that mapped to the early-labeled band on chromosome 1p36. We found that despite their close proximity there was a one-hour difference in the time of activation of the two origins. These studies also documented the approximate location of a replication barrier between the two origins where a replication fork terminates or stalls until replication of the region is completed when a fork progressing from the later activated origin reaches the barrier (Labib and Hodgson, 2007). We used the DNA fiber analysis technique after sequential incorporation of IdU and CldU to show the site and directionality of DNA replication tracks and employed a hybridization probe targeted to this region in 1p36 so that the locations of origins of DNA replication within this region could be determined. The labeling of DNA was studied using several progressively timed intervals of precursor incorporation during the first three hours of S phase (Figure 7). Although we confirmed that the two originally-identified origins were activated in the first and second hours of the S phase, the fiber analysis of DNA replication in this region demonstrated the locations of eight additional origins of replication and some of these origins were activated even earlier. Thus, we have been able to create a temporal map of origin activation over an approximately 400 kb region of 1p36. From these results it appears that replication in this region is organized with activations of small cohorts of temporally-linked consecutive origins and one of these cohorts is consistently activated first.

Neither the locations nor the time of activation of mammalian origins have been linked to the base sequence of DNA (See Mechali, et al. for a recent review (Mechali, 2010)). Consequently the locations of origins and the temporal order of their activation are thought to reside in the epigenetic code specified by chromosomal proteins, including specifically modified histones as well as the DNA methylation pattern. Since the preparation of extended DNA fibers involves the removal of associated proteins this technology does not lend itself to the study of the functional role of epigenetic modifications of chromatin and other associated chromatin proteins in determination of sites that are used as origins or their temporal order of activation. To accomplish this it would be necessary to be able to extend intact chromatin where proteins remain associated with specific sites in the genome.

Analysis of Chromatin Structure and Dynamics of DNA Replication

To accomplish the goal of visualizing chromatin fibers we modified previously reported methods (Quelo and Verbelen, 2004; Sullivan and Karpen, 2004; Lam *et al.*, 2006; Labib and Hodgson, 2007) such that we treated nuclei under less denaturing conditions (as compared to the conditions used to prepare DNA fibers) to release chromatin, and again used the hydrodynamic power of a moving fluid meniscus to straighten individual chromatin fibers (Figure 8). The chromatin fibers that we obtained were eight times more condensed than DNA fibers based on length measurements of equivalent regions obtained with the two methods (Figure 9) (Cohen *et al.*, 2009). Chromatin fibers commonly measured four Mb in length with a maximum of more than 7 Mb. While there are many epigenetic targets that would be interesting to study, along with sequentially incorporated DNA precursors and probes for in situ hybridization, there are a limited number of fluorescence channels that can be discriminated using confocal laser scanning microscopy. Our experiments to date have

only allowed us to detect three fluorescent tags at a time. To associate chromatin proteins with replication we have incorporated one precursor into the DNA and used the other two channels to localize proteins. By examining the relationship between the two proteins at replication sites it was possible to determine their dynamic involvement and interaction in the replication process. For example, proteins of the GINS complex were found with ORC proteins at some pre-replication complexes just prior to the activation of origins (Figure 10) (Cohen *et al.*, 2009). Thereafter the GINS complex remains with the fork during elongation until DNA replication is completed. Therefore, this technology has been used successfully to identify the locations of histones with specific methylation or acetylation modifications as well as the interactions of certain other replication proteins. In the future this technology may play a role in deciphering or confirming the hypothesized “histone code” where multiple histone modifications are presumed to work cooperatively in determining genomic functions (Strahl and Allis, 2000; Turner, 2000; Gilbert, 2002; Vogelaer *et al.*, 2002; Fischle *et al.*, 2003; Kemp *et al.*, 2005; Zhou *et al.*, 2005; Hiratani and Gilbert, 2009). Although the chromatin fiber analysis technique has limited resolving power for detection of single protein molecules in fibers, this shortcoming is offset by its ability to examine the differences and similarities of the locale of proteins in a single fiber.

Summary and Perspective

In summary, recently developed technologies have begun to draw back the curtain of mystery that obscures some of the basic mechanisms of DNA replication at multiple levels. Extended DNA and chromatin fiber have proven valuable for identifying the location of origins of replication at specific genomic sites and determining their temporal order of replication, for identifying and quantifying sites of DNA damage and localizing chromatin proteins in relation to sites of DNA replication. The future potential of these methods include further discoveries in functional genomics and contributions to the elucidation of the histone code. Such studies could prove very valuable in studies of the mechanisms of cancer development, aging, and other processes of disordered genomic functioning.

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Methods to Distinguish Replication Structures on Extended DNA Fibers

Figure 1. Methods to Distinguish Replication Structures on Extended DNA Fibers

Cultured cells were sequentially pulsed with two thymidine analogs. Cells were applied to siliconized glass slides and lysis buffer was applied to the cells. After 10 minutes the slides were rotated to an angle of 25 degrees from the vertical and DNA fibers were allowed to extend by the hydrodynamic force of the fluid flowing down the slide. Following drying of the slides, fluorescent antibody labeling techniques were used to distinguish the first DNA analog, iododeoxyuridine (IdU) from the second DNA analog, chlorodeoxyuridine (CldU) at sites of DNA replication. When viewed in a confocal laser scanning microscope, the immunostaining procedures revealed red fluorescent tracks at DNA replication sites where IdU had been incorporated and green fluorescent tracks where CldU had been incorporated. By examining the relationships between the red tracks and green tracks in images of replicating DNA where both labels were present, it was possible to distinguish four different patterns of labeling following this program of analog incorporation. Tracks where red labeling is flanked on both sides by green labeling are sites where an origin fired within the red labeled segment. Conversely, tracks where red labeling flanks green labeling represents sites where replication in two adjacent replicons converge to a termination site within the green track. Tracks with continuous alternating red and green segments represents clusters of replicons that are spaced closely enough to be completed in the relatively short labeling interval. The tracks with one red segment adjacent to one green segment, which we have called “elongations,” includes several replication structures. These probably include segments of bidirectional replication from origins activated prior to the first labeling interval, some of these arising in clusters of larger replicons, as well as unidirectional replication from origins, or replication sites reaching termini but not simultaneously with the adjacent replicon. Slides also contained red-only and green-only tracks. These tracks were included in the analysis of studies where their presence was interpretable.

Progression of DNA Replication at Origins in Normal and Cancer Cells in First and Fourth Hours of S Phase

Figure 2. Progression of DNA Replication at Origins in Normal and Cancer Cells in First and Fourth Hours of S Phase

In this study normal cells (NHF1) and malignant cells (T98G) were synchronized and labeled sequentially with two distinguishable thymidine analogs starting at the beginning of the first and fourth hours of the S phase. In these studies the first analog was present for 10, 20, or 45 minutes and the second analog was present in all cases for 20 minutes. After extension of DNA fibers the slides were immunostained to identify sites of DNA replication based on the relationship of the two analogs in the labeled tracks (as described in the legend to Figure 1). The replication tracks were observed and photographed using a confocal laser scanning microscope. In these studies only replication tracks that were identified as those of origins of bidirectional replication (red labeling flanked on both sides by green labeling) were scored. The lengths of the red and both green segments of these bidirectional origins were determined under conditions where the observer was blinded to the experimental group of the slides. At the completion of the experiment the coding of the slides was broken and the results were as represented in this Figure (Frum *et al.*, 2008). DNA replication was found to pause between 10 minutes and 20 minutes (the red replication track did not get longer) in normal cells during the first hour of S phase but not during the fourth hour of S phase. Pausing did not occur in the cancer cells.

Figure 3. Percent Distributions of Replication Structures Throughout S Phase

In this study normal cells (NHF1) and malignant cells (T98G) were synchronized and labeled sequentially with the two distinguishable thymidine analogs starting at the beginning of hours one through seven of S phase. DNA fibers were extended and immunostained as described previously. Images of labeled fibers on coded slides were obtained using a confocal laser scanning microscope. Labeled replication tracks were scored as origins, elongations, clusters and terminations as describe in Figure 1. The abundance of each of these patterns were determined at each hour of the S phase for both normal and cancer cells and the percentage of the total was determined for each pattern at each time point. In the graph, data for normal cells are shown with solid lines and data for cancer cells is shown with dashed lines. **(A)** In this panel initiations are shown with black lines while terminations are shown with gray lines. **(B)** In this panel elongations are shown with black lines while clusters are shown with gray lines. The extensive statistical evaluation of the data obtained and the variance of observations were reported in the original publication (Frum *et al.*, 2009).

Average Lengths of DNA Tracks in Each Hour of S Phase

Figure 4. Average Lengths of DNA Tracks in Each Hour of S Phase

In the study reported in Figure 3, length measurement of labeled segments were made during the assessment of replication tracks. In this graph the length of all labeled tracks from origins, elongations, clusters and terminations were averaged for each hour of the S phase. For all hours except the first, the length of the label tracks was longer in the normal cells than the cancer cells. The one exception is the first hour of S phase where the track length is shorter in normal cells, presumably as a consequence of pausing near origins activating in this interval.

Detection of AP sites on DNA Fibers **Figure 5. Detection of AP sites on DNA Fibers**

This black and white version of a color photograph shows the distribution of apyrimidinic / apurinic sites in extended DNA fibers. Cultured cells were treated with H_2O_2 and then cells were applied onto slides and DNA fibers were extended by the force of a receding meniscus. Thereafter, DNA fibers were stained with the fluorescent dye YOYO. In the original photograph the DNA fibers are stained green (but here are seen as white lines). The AP sites that were present were labeled with a biotin-tagged aldehyde reactive probe which were made to fluoresce as red dots (here their localization has been enhanced by marking them with white dots with black borders). As can be seen in the photo, AP sites were often closely clustered.

Quantitation of AP Site Formation

Figure 6. Quantitation of AP Site Formation

The number and distribution of AP sites were determined in conjunction with measurements of the lengths of DNA fibers. From this data the number of AP sites per 10⁶ nucleotides was determined. **(A)** In this panel the measurement of AP sites per 10⁶ nucleotides made by standard slot blot assay is compared to the results found using DNA fiber analysis. In this experiment, cells were either untreated (control) or treated with H₂O₂ and extended DNA fibers were prepared from both. DNA fibers were stained green with YOYO and AP sites were detected using ARP yielding bright red dots on the DNA fibers (see Figure 5). The number of AP sites per 10⁶ nucleotides was determined by analysis of images of the fibers obtained by confocal laser scanning microscopy that measured the lengths of DNA segments and counted AP site signals along these segments. In parallel, the number of AP sites per 10⁶ nucleotides was determined by the standard slot blot analysis technique (Nakamura *et al.*, 1998; Chastain *et al.*, 2010). In this chart the observations from controls are illustrated with the dark dotted columns and the higher observations from H₂O₂-treated cells are illustrated with lighter gridded columns that are behind the control columns. The variance of the measurements is reflected by the error bars on each column. It should be noted that AP sites in controls are caused by reactive oxygen species generated by natural cellular processes including metabolic processes and may result from naturally formed H₂O₂ as well as other metabolic products. The column for the H₂O₂-treated cells shows the increment in AP sites resulting from the treatment. The measurements of AP sites per 10⁶ nucleotides were not statistically distinguishable but the variance in measurements was lower using the fiber analysis technique. **(B)** In this panel DNA at sites of replication were marked by sequential treatments with two thymidine analogs and cells were sham treated or treated with 20 μ M H₂O₂ between exposures to the first (IdU) and second (CldU) thymidine analogs. In this panel the observations from sham-treated cells are in the left columns and the observations from the H₂O₂-treated cells are in the right columns. The dark dotted columns show the AP sites in segments of DNA tagged with the IdU and the higher observations in segments of DNA tagged with CldU are illustrated with lighter gridded columns that are behind the IdU columns. These results show that AP sites are more abundant in replicating DNA and that this effect is greatly enhanced by treatments of cells with exogenous H₂O₂ (Chastain *et al.*, 2010).

Figure 7. Organization and Temporal Order of DNA Replication in the 1p36 Region

(A) In this panel a cosmid clone from a library of early replicating human DNA (shorter line on the left) and a BAC (longer line) are shown mapped to chromosome 1p36. An origin of DNA replication activated in the first hour of S phase had been mapped to the cosmid. (B) In this panel we show a composite of black and white photographs from DNA fibers derived from color photos of fibers labeled with the two analog sequential labeling technique discussed above. The photomicrograph of the FISH probe is shown at top below the drawing of the FISH probe location. Below that image is a photo of a fiber illustrating both the FISH probe and DNA precursor labeling. The three lower photographs are images of DNA replication labeling in fibers observed following labeling in the intervals 20-50 minutes into the S phase, between 5-35 minutes of the second hour, and between 5-35 minutes of the third hour of S phase. The DNA labeling for 5-35 minutes of the second hour is part of the composite image labeled as "FISH probe and DNA analogs." This shows that different regions of this segment of the genome are replicated at different times into the S phase. (C) In the top part of this panel the locations of observed DNA replication labeling sites in these three intervals of S phase are presented in a drawn representations. Below these, the deduced order of replication through this region is drawn with gray shades corresponding to the observations in the upper part of this panel; replication occurs first in the lightest shaded part of the line and the shade is darker as the time of replication progresses later into the S phase. At the bottom of this panel are the sites of the origins of replication and the presumed direction of replication in this region based on observations in colored images with two sequential analog labeling. The temporal order of replication, the sites of origins, and the directions of replication are scaled to the genomic position scale in panel A. Replication appears to be occurring through replication of cohorts of roughly contemporaneously-activated replicons.

Methods for Preparing Extended Chromatin Fibers

Figure 8. Methods for Preparing Extended Chromatin Fibers

Unsynchronized normal NHF1 cells were labeled briefly with the thymidine analog EdU prior to harvesting of the cells. The cells were treated with hypotonic buffer to burst the cell membranes and then were cytopsin onto microscope slides. Lysis buffer with the DNA dye DAPI was dropped onto the slide and a cover slip was placed atop the drop of lysis buffer. The hydrodynamic force of the meniscus formed as the coverslip excluded fluid and came into contact with the slide provided the means of extending the chromatin fibers on the slide. Thereafter, the chromatin fibers were immuno-stained with antibodies to proteins of interest followed by appropriate washings and then they were fixed. Next the EdU was treated with reaction buffer to fluorescently label the sites of newly replicated DNA. In some cases the final step was to stain DNA with YOYO. Stained slides were examined in a confocal laser scanning microscope to identify images consistent with single chromatin fibers and these images were photographed for later analysis.

Calibration of DNA and Chromatin Fiber Lengths

To determine the number of kilobases of DNA per micron in DNA fibers, the lengths of FISH probes on straightened and aligned DNA fibers from NHF1 cells were measured. FISH probe hybridized to DNA fibers from human chromosome 1 (13 measurements total, average length = 1.9 kb/ μ m).

The difference in the degree of compaction between DNA fibers and extended chromatin fibers was determined by labeling NHF1 cells for 20 min with 30 μ M IdU (DNA Fibers) or EdU (Chromatin Fibers) and measuring track lengths. These studies demonstrated that the extended chromatin fibers used in our studies are 8.3 times more compact than our aligned DNA fibers and contain approximately 15.9 kb of DNA per micron of length.

Figure 9. Calibration of DNA and Chromatin Fiber Lengths

The study illustrated in this Figure shows how the lengths of extended DNA fibers and extended chromatin fibers are related. Using the known genomic length (240 kb) of the DNA marked by a FISH probe and the several measurements of the length of FISH probe images it was determined that these fiber lengths corresponded to 1.9 kb/ μ m. Thereafter cells were treated for 20 minutes with either IdU (to label DNA fibers) or EdU (to label chromatin fibers). The averages of several measurement of the lengths of labeled DNA tracks were determined for the DNA and chromatin fibers. Since the lengths of tracks labeled for the same interval was 32 μ m for DNA fibers and 3.8 μ m for chromatin fibers we determined that the chromatin fibers are 8.3 times more compact than the DNA fibers.

Figure 10. Chromatin fiber studies of DNA Replication

Chromatin fibers were prepared and stained as previously described (Cohen *et al.*, 2009; Cohen *et al.*, 2010). **(A)** Distribution of ORCs (red), GINS (blue) in relation to active sites of DNA replication (EdU, green) on an extended chromatin fiber. On this fiber several stages of the replication process can be seen: 1) regions of ORC1 binding, 2) a region of ORC1 and PSF1 binding prior to the start of DNA replication, 3) regions of ORC1 and PSF1 binding at sites of active site of DNA replication, 4) a region that has completed DNA replication, ORC1 and PSF1 are no longer bound. **(B)** Distribution of the SWI/SNF protein BRG1 (red) and acetylated Histone 3 (H3K9ac) (blue) in relation to sites of DNA replication (EdU, green). Two sites where there is overlap of BRG1 and H3K9ac at sites of replication are indicated (arrows). We reported previously that 87% of sites where H3K9ac and EdU co-localize also had BRG1 (Cohen *et al.*, 2010). Bars $\cong 25 \mu\text{m}$ ($\cong 400 \text{ kb}$; bottom right of each panel).