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Metazoan Origins of DNA Replication: Regulation Through Dynamic Chromatin Structure

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ABSTRACT

DNA replication in eukaryotes is initiated at multiple replication origins distributed over the entire genome, which are normally activated once per cell cycle. Due to the complexity of the metazoan genome, the study of metazoan replication origins and their activity profiles has been less advanced than in simpler genome systems. DNA replication in eukaryotes involves many protein–protein and protein–DNA interactions, occurring in multiple stages. As in prokaryotes, control over the timing and frequency of initiation is exerted at the initiation site. A prerequisite for understanding the regulatory mechanisms of eukaryotic DNA replication is the identification and characterization of the *cis*-acting sequences that serve as replication origins and the *trans*-acting factors (proteins) that interact with them. Furthermore, in order to understand how DNA replication may become deregulated in malignant cells, the distinguishing features between normal and malignant origins of DNA replication as well as the proteins that interact with them must be determined. Based on advances that were made using simple genome model systems, several proteins involved in DNA replication have been identified. This review summarizes the current findings about metazoan origins of DNA replication and their interacting proteins as well as the role of chromatin structure in their regulation. Furthermore, progress in origin identification and isolation procedures as well as potential mechanisms to inhibit their activation in cancer development and progression are discussed. J. Cell. Biochem. 106: 512–520, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: INITIATION OF DNA REPLICATION; REPLICATION ORIGINS; ORIGIN ACTIVITY; CHROMATIN STRUCTURE; ORIGIN BINDING PROTEINS

Metazoan DNA replication is a tightly regulated process, ensuring that the genome is duplicated only once before chromosome segregation and cytokinesis. There are approximately 10^4–10^6 replication origins each spaced apart at approximately 50–250 kb, depending on the stage of development, growth conditions, or cell transformation status [Edenberg and Huberman, 1975; Hand, 1978; Martin, 1981; Anglana et al., 2003], that are coordinately activated along the chromosomes to ensure that the entire genome is replicated only once per cell cycle [Blow and Dutta, 2005; Machida et al., 2005]. The initiation of eukaryotic DNA replication is divided into two stages: origin selection and origin activation. Origin selection is mediated by the assembly of the pre-RC [Mendez and Stillman, 2003], which initiates with the binding of the *o*rigin *r*ecognition *c*omplex (ORC) [Chesnokov, 2007; Sasaki and Gilbert, 2007] onto origin DNA. During G_1, the ORC recruits other members of the pre-RC, including the proteins Cdc6 and Cdt1 and the putative DNA helicase, the minichromosome maintenance complex (MCM2-7). Once the origins are thus selected, they are licensed by the action of cyclin-*d*ependent *k*inases (Cdks) and the Dbf4-dependent kinase, ushering the cells into S-phase [Sclafani and Holzen, 2007]. Together these kinases trigger the recruitment of replication proteins necessary for origin unwinding and DNA synthesis. This review examines the methods employed for the identification and isolation of eukaryotic origins, the features of these origins, as well as some of the known origin binding proteins.

ISOLATION METHODS OF EUKARYOTIC REPLICATION ORIGINS

Several experimental approaches have been employed over the years for the visualization and isolation of eukaryotic replication origins [reviewed in Vassilev and DePamphilis, 1992]. These include: (a) methods based on analyses of nascent strands, such as DNA fiber autoradiography, earliest labeled DNA fragment, replication origin trapping, nascent strand extrusion, nascent strand length, and replication fork polarity; (b) methods based on analyses of DNA structures, such as electron microscopy and the

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two-dimensional (2D) gel electrophoresis; and (c) methods based on functionality, such as ARS assays in yeast and in mammalian cells. Although these techniques have been instrumental in identifying replication origins in a variety of multicellular organisms, they are not suitable for genome-wide analyses of complex genomes, leading to the identification of only a small portion of replication origins [Todorovic et al., 1999].

A recent cloning strategy for sequences comprising mammalian replication origins exploited an origin-trapping assay, whereby replication bubbles are selectively retained (trapped) in agarose due to their circular nature [Mesner et al., 2006]. The assay described was based on the "agar fixation" principle according to which, when a mixture of linear and circular DNA is subjected to electrophoresis the linear fragments migrate through the gel whereas the DNA circles are topologically linked to the agarose mesh [Wada and Kishizaki, 1968, #9331]. Using this procedure, Mesner et al. were able to yield highly purified preparations of replication bubbles and generated a replication origin library from S phase Chinese hamster ovarian cells. The development of this new systematic screening tool for large-scale identification of origin sequences opens the door for the better understanding of the characteristics and regulation of metazoan origins of DNA replication.

FEATURES OF REPLICATION ORIGINS

LOCAL CHROMATIN STRUCTURE

The chromatin environment influences both replication timing and frequency of origin activation [Aladjem, 2007]. Chromatin exists in either decondensed (euchromatin) or condensed (heterochromatin) state. Euchromatin corresponds to genomic regions that are decondensed during interphase and contain either actively transcribing genes or potentially active ones [Dillon and Festenstein, 2002; Kosak and Groudine, 2002]. The regulatory sequences in these regions are accessible to nucleases, commonly have unmethylated CpG islands, and the core histones H3 and H4 are hyper-acetylated on their N-terminal lysine residues [Zhang et al., 2005; Benson et al., 2006]. Regulation of chromatin structure occurs through post-translational modifications of the histone amino-terminal tails, including acetylation, methylation, phosphorylation, ubiquitylation and sumoylation [Kouzarides, 2007]. Alternatively, ATP-dependent chromatin remodeling factors alter histone–DNA interactions, so that proteins can interact with nucleosomal DNA. These modifications enable a dynamic chromatin state, in which diverse nuclear processes can occur systematically [Jenuwein and Allis, 2001; Cosgrove, 2007; Ruthenburg et al., 2007].

Replication initiates at earlier times in S-phase at euchromatic as opposed to heterochromatic regions [Woodfine et al., 2004; Jeon et al., 2005], with certain exceptions [Kim et al., 2003; Prioleau et al., 2003]. In mammalian cells, treatment with the histone deacetylase inhibitors (HDACi) sodium butyrate and Trichostatin A (TSA), but not with the DNA methylation inhibitor azacytidine, changed the replication timing imprint of intra- and inter-chromosomal loci, indicating the importance of chromatin structure in determining the replication program [Bickmore and Carothers, 1995]. Similarly, treatment of HeLa cells with TSA changed the selection of replication initiation sites from a more localized into a more dispersive pattern and led to earlier firing of specific origins [Kemp et al., 2005]. Altogether, these studies suggest a role for epigenetic regulation of both origin selection and temporal activation.

These dynamic properties of chromatin structure arise from the action of multiple nuclear proteins that modulate its configuration and function. For example, deletion of the budding yeast histone deacetylase Rpd3 increased histone acetylation levels at many replication origins causing them to fire earlier in S phase [Aparicio et al., 2004]. Similarly, targeting the histone acetylase Gcn5 to a late origin, induced an "open chromatin" status and accelerated its activation timing [Vogelauer et al., 2002]. In *Drosophila melanogaster* amplification-stage follicle cells, chromatin at the origin associated with the chorion locus is hyperacetylated during gene amplification, while inactivation of the dRpd3 histone deacetylase led to genome-wide hyperacetylation, genomic replication and redistribution of the origin recognition complex (ORC). Similarly, tethering dRpd3 or the Chameau acetyltransferase to the chorion locus affected its replication activity suggesting the importance of epigenetic factors on origin activity in metazoa [Aggarwal and Calvi, 2004].

In addition to histone modifications, nucleosome repositioning is involved in general chromatin remodeling events. One of the ATP-dependent chromatin remodeling complexes, ACF1-SNF2h, is localized to peri-centromeric heterochromatin during its replication in late S phase [Collins et al., 2002]. Cells depleted of ACF1 demonstrate delayed progression of replication in late S phase, indicating that ACF1-SNF2h is required for DNA replication at heterochromatic regions. The replication defect of ACF1-depleted cells was rescued by 5-aza-2-deoxycytidine treatments, which caused decondensation of heterochromatin through inhibition of DNA methylation. Thus, it appears that the ACF1-SNF2h complex is required for the remodeling of heterochromatin prior to its replication. Although it is not clear whether SNF2h plays a role in chromatin remodeling at replication origins or forks, it is known to be recruited to remodel chromatin at the Epstein-Barr virus (EBV) origin, where host cell initiation machinery is utilized [Zhou et al., 2005]. Similarly, another ATP-dependent chromatin remodeling complex, NoRC, associates exclusively with late- but not early-replicating ribosomal RNA genes (rDNA). NoRC overexpression resulted in epigenetic silencing of the chromosomal locus as well as resetting of the replication time of early-replicating rDNA arrays from early to late [Li et al., 2005].

CRUCIFORM STRUCTURES

Cruciforms are naturally occurring DNA secondary structures which arise through intra-strand base pairing of palindromic DNA sequences. Such structures are widely distributed in the DNA of both prokaryotes and eukaryotes [Wilson and Thomas, 1974; Schmid et al., 1975; Panayotatos and Fontaine, 1987] and may affect the supercoiling degree of DNA, nucleosome positioning, formation of other DNA secondary structures or directly interact with proteins [reviewed in Pearson et al., 1996]. Numerous studies have shown that cruciforms serve as recognition signals at or near origins of DNA replication [Pearson et al., 1996; Zannis-Hadjopoulos et al., 2004]. Using monoclonal antibodies (mAbs) raised against cruciform DNA structures it was shown that there is a dynamic formation

of cruciforms in mammalian nuclei, reaching a maximum at the G_1/S boundary [Ward et al., 1990; Ward et al., 1991]. Furthermore, use of these mAbs led to the purification of active mammalian origins of replication [Bell et al., 1991], whereas their addition to permeabilized cells resulted in a two- to sixfold enhancement of DNA synthesis [Zannis-Hadjopoulos et al., 1988]. Altogether, these results support the notion that cruciform structures play a critical role in the initiation of DNA replication by a mechanism that is thought to involve cruciform stabilization and recognition by replication initiator proteins. In support of this, a cruciform-binding activity was purified which was identified as a member of the 14-3-3 protein family and participates in the initiation of DNA replication [Pearson et al., 1994b; Todd et al., 1998; Yahyaoui et al., 2007; reviewed in Zannis-Hadjopoulos et al., 2008].

By analogy to DNA replication, cruciforms are also found in transcription regulatory elements in the genomic DNA of both prokaryotic and eukaryotic species where they have been involved in the regulation of gene expression [Kim et al., 1998; Lienard et al., 2006]. Therefore, it has been proposed that under certain physiological conditions inverted repeat sequences can extrude from the linear to the cruciform conformation and serve as recognition signals for specific DNA replication and transcription factors [Zannis-Hadjopoulos et al., 1984; Sinden, 1994; Pearson et al., 1996; Wadkins, 2000].

CONSENSUS SEQUENCES IN DNA REPLICATION ORIGINS

Previous attempts to determine a consensus sequence for replication origins through computer-based analyses of chromosomal DNA sequences harboring initiation sites resulted in the identification of a 21 bp sequence, but it was not determined whether that sequence conferred autonomous DNA replication origin activity [Dobbs et al., 1994]. A recent study, reported a site-specific interaction of the murine pre-RC with a short repeated DNA element within the replication origin of the rDNA locus, suggesting that short DNA sequences are crucial for origin recognition [Zellner et al., 2007]. Supporting this notion, a short (36 bp) putative mammalian consensus sequence that designates replication initiation sites (origins) had been identified earlier, using four mammalian autonomously replicating sequences containing α-satellite DNA and a reiterative process between pairs of African green monkey and human sequences to minimize derivation of a α-satellite consensus [Price et al., 2003]. This consensus sequence was capable of supporting autonomous replication of a plasmid after transfection into eukaryotic cells. Initiation of DNA replication occurs within the consensus and homologues of it are found consistently at mammalian chromosomal sites of initiation and within CpG islands, which coincide with replication initiation sites [Delgado et al., 1998; Price et al., 2003]. Versions of the consensus sequence are found at known sites of initiation of DNA replication, including the γ-aminobutyric acid receptor subunit β3 and α5 gene cluster [Sinnett et al., 1996; Strehl et al., 1997] and at the *dnmt*1 (human DNA methyltransferase) locus [Araujo et al., 1999], and function as origins of DNA replication in normal and malignant human cells, immortalized monkey and mouse cells, and normal cow, chicken and fruit fly cells. Mutation analysis of the 36 bp consensus sequence indicated that an internal 20-bp human consensus sequence (20mer) is sufficient to act as a core origin element. The distribution of this 20mer consensus over 1 Mb of human chromosomal DNA is similar, quantitatively and qualitatively to the distribution of the ARS consensus sequence (ACS) on *S. cerevisiae* chromosomes [Price et al., 2003]. Finally, six versions of the 20mer which were analyzed ectopically and endogenously were found to act as origins of DNA replication in plasmids as well as in situ, at their chromosomal loci, suggesting that the 20-bp consensus sequence can be used to predict chromosomal regions that contain replication origins [Di Paola et al., 2006].

ORIGIN BINDING PROTEINS

Origin recognition proteins bind specifically to a site within the replication origin and participate in the initiation of DNA replication either directly by unwinding the DNA (helicase activity), or indirectly through their interaction with other replication proteins [DePamphilis et al., 2006; Sclafani and Holzen, 2007].

PRE-REPLICATION COMPLEX (PRE-RC)

Replication origins are marked by: (a) the presence of a mammalian consensus sequence (Consensus Sequences in DNA Replication Origins Section) within a favorable chromatin context, and (b) the binding of ORC, which acts as a cell-cycle-regulated landing dock for the downstream initiator proteins Cdc6 and Cdt1 (Fig. 1). Binding of the latter two proteins during G_1-phase permits the subsequent loading of the putative DNA helicase, the minichromosome maintenance protein complex (MCM2-7) [Cook et al., 2004], forming the pre-replication complex (pre-RC), and thus licensing the replication origins [Sclafani and Holzen, 2007; and references therein]. The MCM2-7 complex has very low helicase activity in vitro by itself [Ishimi, 1997; Lee and Hurwitz, 2000], but it is active when complexed with cdc45 and GINS [Moyer et al., 2006]. At the G_1/S transition, the activity of two kinases, the Dbf4-dependent kinase

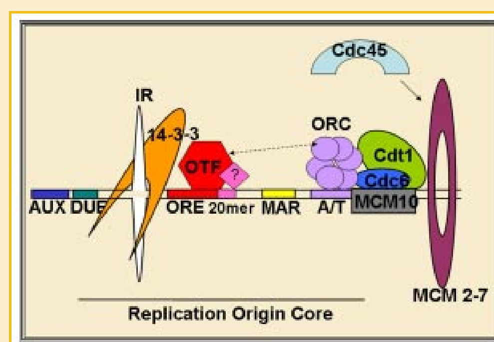

Fig. 1. Model showing the binding of the various initiator proteins at a metazoan origin of DNA Replication. AUX: auxiliary element, DUE: DNA unwinding element, ORE: origin recognition element, 20mer: 20-bp consensus sequence element, MAR: matrix attachment region, A/T: A–T-rich sequence, OTF: ORC targeting factor. The question mark indicates initiator proteins, not identified yet, which may bind to the ORE/20mer. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

(DDK) (Cdc7-Dbf4) and the cell-cycle dependent kinase 2 (Cdk2-Cyclins E/A), results in the formation of a pre-initiation complex, containing the cdc45 and GINS proteins, as well as, the activation of the putative DNA helicase and the recruitment of the replicative DNA polymerases [Mimura and Takisawa, 1998; Tanaka and Nasmyth, 1998; Sheu and Stillman, 2006; Yabuuchi et al., 2006; Krasinska et al., 2008]. Upon entry into S phase, multiple mechanisms ensure that the replication initiation machinery is inactivated so as to avoid re-replication of chromosomal regions and genome instability [Blow and Dutta, 2005; Hook et al., 2007]. In yeasts, a number of S-phase CDK-dependent events prevent re-licensing of replicated DNA, which include: (i) Cdc6 phosphorylation at the G_1/S transition which targets it for degradation [Elsasser et al., 1999; Drury et al., 2000]; (ii) decrease of the *Schizosaccharomyces pombe* Cdt1 (SpCdt1) levels during S-phase, possibly by a CDK-mediated degradation mechanism [Nishitani et al., 2000]; and (iii) inactivation of the ORC complex by CDK phosphorylation [Vas et al., 2001]. An alternate mechanism of pre-RC inactivation after origin firing was also described in *S. cerevisiae*, involving the direct interaction of CDKs with ORC6 and Cdc6, which inhibits pre-RC assembly in S phase [Mimura et al., 2004; Wilmes et al., 2004]. In metazoans, ORC1, the subunit of ORC that has ATPase activity, is targeted for degradation in S-phase by a SCF (Skp2)-dependent ubiquitination reaction [Mendez et al., 2002], while human Cdc6 is exported from the nucleus due to the CDK enzymatic activity, which may play a role in preventing re-replication [Saha et al., 1998; Petersen et al., 1999]. The dominant pathway that is functioning in preventing metazoan origin re-licensing, however, involves the blockage of Cdt1 activity. Geminin, a specific inhibitor of Cdt1, has emerged as a key regulator of metazoan replication (conserved from *C. elegans* to humans) and Cdt1 activity. Geminin is destabilized during G_1 phase and accumulates during S, G_2 and M phases of the cell cycle, binding directly to Cdt1 and preventing the loading of the MCM2-7 complex to it [Yanagi et al., 2002; Cook et al., 2004]. Furthemore, phosphorylation of Cdt1 by CDK at the G_1/S transition triggers its degradation by the proteasome, through Skp2-dependent and -independent pathways [Liu et al., 2004; Nishitani et al., 2004; Thomer et al., 2004; Arias and Walter, 2005]. Recently, a new evolutionary conserved back-up mechanism was described in the case of re-replication-induced DNA damage; geminin depletion as well as overexpression of Cdt1 or Cdc6 causes re-replication, which might ultimately lead to tumorigenesis. However, re-replication induced by such manipulations is incomplete suggesting the existence of backup events, which restrain re-replication once it begins. Studies in both *Drosophila* and human cells suggest that Cdt1 and Cdc6 ubiquitination and degradation are coupled with geminin overexpression, providing a salvage pathway and minimizing the extent of re-replication and genomic instability [Mihaylov et al., 2002; Ballabeni et al., 2004; Hall et al., 2008].

ORC TARGETING FACTORS

Origin determination relies on the recruitment of the initiator protein ORC, as expression of a GAL4-ORC fusion protein leads to the creation of a functional artificial origin at the GAL4 DNA-binding site [Takeda et al., 2005]. Although the metazoan ORC exhibits virtually no sequence-specificity [Vashee et al., 2003; Remus et al., 2004], unlike the *S. cerevisiae* ORC (*Sc*ORC), the replication initiation events are nonetheless not random, as determined by studies on the lamin B2, c-myc and β-globin origins [Ghosh et al., 2004; Paixao et al., 2004; Wang et al., 2004]. It has been proposed that targeting of metazoan ORC to specific replication origins, relies on the presence of permissive local chromatin structure [Danis et al., 2004 and Pre-Replication Complex (Pre-RC) Section], DNA topology [Falaschi et al., 2007] and/or accessory targeting factors that exhibit sequence-specificity [Bell and Dutta, 2002; Gerbi et al., 2002; Kearsey and Cotterill, 2003]. Remus et al. [2004] showed that *Dm*ORC exhibits mild sequence specificity, but strong preference for negatively supercoiled DNA, suggesting that the topological state of DNA is a critical factor for origin specification in *D. melanogaster*. Recently, topoisomerases I and II were also found to interact specifically with the human lamin B2 replication origin in a cell-cycle-dependent manner, indicating that the role of DNA topology during pre-RC assembly may be applicable in mammalian genomes as well [Abdurashidova et al., 2007; Falaschi et al., 2007].

Another proposed mechanism for metazoan origin specification involves the function of accessory proteins that target ORC to replication origins. The Epstein-Barr virus (EBV) replicates its genome from the latent origin of replication, oriP, using the ORC of the host. Initiation of DNA replication from oriP is affected by the viral transactivator protein EBNA-1 [Schepers et al., 2001], the telomere repeat factor 2 (TRF2) [Atanasiu et al., 2006] and the high mobility group protein A1a (HMGA1a) [Thomae et al., 2008], which have been proposed to recruit human ORC (*Hs*ORC) to the replicator by binding to specific DNA sequences (EBNA-1, TRF2) or the minor groove of AT-tracks (HMGA1a) at chromosomal origins. In mice, the transcriptional repressor protein A1F-C was shown to be able to recruit Orc1 to the rat aldolase B origin, thus effecting replicator activity from this origin [Minami et al., 2006]. Finally, a role for ORC (*Hs*ORC) positioning was also described in humans; human cells hypomorphic for the Ku DNA repair protein displayed decreased origin usage and prolonged G_1 phase due to defective ORC assembly [Sibani et al., 2005a,b], while Ku depletion led to the activation of a replication stress checkpoint [Rampakakis et al., 2008]. A direct, transcription-independent role for c-Myc in replication initiation involving replication origin binding and pre-RC interaction was also recently proposed [Dominguez-Sola et al., 2007].

The above data suggest a model in which ORC can be recruited to multiple sites in the metazoan genome through association with a number of different sequence-specific accessory factors in the presence of permissive local chromatin structure and DNA topology. Such a model would explain the initiation of DNA replication from chromosomal sites with variable DNA sequence as well as the degenerate nature of the metazoan replication origin consensus sequence.

CRUCIFORM BINDING PROTEINS (CBP/14-3-3)

The cruciform *b*inding *p*rotein (CBP) was initially purified via its ability to specifically interact with cruciform DNA regardless of its sequence [Pearson et al., 1994a]. The interaction of CPB with DNA was localized to the four-way junction at the base of the cruciforms, binding onto the elbows of the junctions in an asymmetric fashion

[Pearson et al., 1995]. By microsequencing, CBP was subsequently identified as a member of the 14-3-3 protein family [Todd et al., 1998].

The 14-3-3-protein family is a highly conserved family of 28-33kDa acidic proteins, which are expressed in a range of tissues and are found in all eukaryotic species [Aitken et al., 1992; Wang and Shakes, 1996; Ferl et al., 2002]. The human genome contains at least seven distinct 14-3-3 genes, giving rise to nine isoforms (α, β, γ, δ, ε, ζ, η, σ, and τ, with α and δ being phosphorylated forms of β and ζ, respectively), together with other non-functional pseudogenes [Aitken et al., 1992; Fu et al., 2000]. 14-3-3 isoforms are linked to control of a wide spectrum of biological processes, including cell cycle progression, signal transduction, apoptosis [reviewed in Tzivion et al., 2001; Dougherty and Morrison, 2004; Mackintosh, 2004], DNA replication [reviewed in Zannis-Hadjopoulos et al., 2008], and nucleo-cytoplasmic shuttling of proteins such as class II histone deacetylases [McKinsey et al., 2001]. The isoforms are largely identical, but contain a few regions of diversity. Most of the isoforms are expressed in all tissues, although 14-3-3σ expression is restricted to epithelial cells and τ to T cells [Aitken et al., 2002].

The CBP complex contains the 14-3-3 isoforms β, γ, ε, ζ, and σ [Alvarez et al., 2002] which form specific heterodimers as shown by the ability of 14-3-3 ζ to dimerize only with β, ε, and ζ but not with γ and σ [Alvarez et al., 2003]. This limitation in heterodimer formation is believed to confer specificity on 14-3-3 function [Aitken et al., 2002]. The crystal structure of the 14-3-3 dimers revealed a U-shaped molecule, the center of which binds onto the cruciform [Liu et al., 1995]. CBP/14-3-3 binds in vivo to replication origins in mammalian and *S. cerevisiae* cells in a cell cycle-dependent manner, peaking at late G_1 and decreasing thereafter [Callejo et al., 2002; Novac et al., 2002; Yahyaoui et al., 2007]. With regards to its mechanism of action, CBP/14-3-3 is believed to act as sensor of transient cruciform extrusion during G_1 phase (ORC Targeting Factors Section) serving as attachment site for initiator proteins [reviewed in Zannis-Hadjopoulos et al., 2004; Zannis-Hadjopoulos et al., 2008]. In support of this model, a number of proteomic studies have shown the association of 14-3-3 with initiator proteins and members of the pre-RC complex, among which, the MCM helicase, Ku, Replication Factor C and DNMT1 [Meek et al., 2004; Pozuelo Rubio et al., 2004; Satoh et al., 2006]. A model showing the interaction of the various initiator proteins with replication origins is shown in Figure 1.

DNA REPLICATION AND CANCER

ORIGIN ACTIVITIES IN NORMAL VERSUS TRANSFORMED/TUMOR CELLS

The activities of DNA replication origins over a 12.5 kb region of the human c-myc locus were shown to be twofold higher in HeLa cells as in NSF cells, suggesting that cell transformation may induce greater frequency of initiation of origins at certain loci [Tao et al., 1997, 2000]. A twofold increase in origin activity across the same 12.5 kb region of the human c-myc locus was also found in the isogenic cell lines WI38 and their transformed counterpart WI38 (SV40) [Tao et al., 2001], ruling out the possibility that cell type effects were the cause of the differential origin usage found in HeLa and NSF cells. It was thus concluded that the increased origin activities were the result of cellular transformation.

More recent studies have found two- to threefold higher origin activities in transformed/tumor cells compared to normal cells, suggesting higher activation of these origins located across this ~211 kb region on human chromosome 19q13 [Di Paola et al., 2006]. Again, use of the isogenic cell lines of WI38 and WI38(SV40) showed that the origins are at least twice as active in the transformed cell line (WI38(SV40)) compared to that of its normal counterpart (WI38), ruling out the possibility that cell type effects were responsible for increased frequency of initiation. The increased origin activity across the ~211 kb region in tumor/transformed cells compared to normal cells may be influenced by one or many parameters regulating DNA replication, such as the concentration and conformation of initiator proteins [McNairn and Gilbert, 2005; Lau et al., 2007; Blow and Gillespie, 2008], specific DNA sequences within the initiation sites having differential affinities for ORC [Bell, 2002], gene transcription [DePamphilis, 1993], chromatin structure [Melendy and Li, 2001; Aladjem, 2007], nuclear organization [Taddei et al., 2004; Ottaviani et al., 2008], and nucleotide pool levels [Anglana et al., 2003]. Previous work suggests that there are at least two types of malignant changes in regulation of DNA replication, the unexpected increase in origin activity at some loci [Tao et al., 1997, 2000, 2001; Di Paola et al., 2006] and activation of silent origins [Martin and Oppenheim, 1977; Oppenheim and Martin, 1978]. Thus, it appears that there are at least three subsets of origins; those that are normal and remain unchanged, those with increased activity in immortalized or malignant cells, and those that are activated solely in tumor cells.

POTENTIAL ROLE OF REPLICATION IN TUMORIGENESIS

How replication origins participate in oncogenesis is of considerable interest due to their widespread roles in tumorigenesis and/or tumor suppression. Recent findings from the INK4/ARF locus [encoding for the tumor suppressors p15(INK4b), ARF and p16(INK4a)], one of the most frequently inactivated loci in human cancer [reviewed in Sherr, 2000], shed light on this process. A putative DNA replication origin was identified at the INK4/ARF locus that coincides with a conserved noncoding DNA element (regulatory domain RD(INK4/ARF) and assembles a multiprotein complex containing Cdc6, Orc2 and MCMs [Gonzalez et al., 2006]. Cdc6 overexpression resulted in targeted and localized heterochromatinization of RD(INK4/ARF), as well as, transcriptional repression of the locus, demonstrating that RD(INK4/ARF) is a relevant transcriptional regulatory element. The authors concluded that this mechanism was consistent with the silencing of the mating-type HM loci in yeast by replication factors. In addition to the ability of Cdc6 to repress the INK4/ARF locus, the c-Myc proto-oncogene has gained considerable attention as controlling DNA replication [Cole and Cowling, 2008]. Dominguez-Sola et al. reported that c-Myc promotes DNA replication via a nontranscriptional mechanism, and that c-Myc deregulation causes DNA damage predominately during S phase. These results establish c-Myc as a new DNA replication factor and suggest an alternative model for its role in cell growth and tumorigenesis [Dominguez-Sola et al., 2007].

PERSPECTIVES

The ability to assess the dynamics of DNA replication at a genome-wide level has provided new insights into the global regulation of DNA replication. Different approaches, such as replication timing and genome-wide localization analyses, have been used to identify replication origins and characterize their temporal pattern of activation in a variety of eukaryotic cell types. Such studies have revealed that origin specification is more complex than was previously thought, with multiple parameters being important, such as specific *cis*-acting sequences recognized by one or more trans-acting factors, DNA secondary structure, chromatin structure and epigenetic regulation. In the long term, the studies of replication origin identification will have to be combined with genetic and biochemical analyses in order to fully characterize the key determinants of origin function and gain a better understanding of the mechanisms regulating the initiation of DNA replication. However, the availability of large numbers of DNA sites that act as origins of replication will be essential for these studies to determine the mechanisms that direct pre-RC formation and origin activation in metazoa. Although all eukaryotic organisms maintain a clear pattern of replication timing across their genome, the mechanisms responsible for establishing this conserved and characteristic pattern remain obscure. Understanding of the intricate mechanisms regulating DNA replication will depend on uncovering the interplay between all the known parameters involved as well as identifying new ones. With regard to the changes in DNA replication that result from the stepwise progression toward tumorigenesis, the ultimate goal will be to develop a sensitive detection method for cells at an early step of malignant transformation. It will also be vital to discover factors that are required for the activation of tumor-specific origins of DNA replication in order to develop a gene vector therapy system, specific for predisposed (immortal, but not yet tumorigenic) and malignant cells, that will allow their specific targeting.

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