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DNA Replication in Mammals

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Formulated in the early 1960s, the replicon hypothesis proposed that DNA synthesis is controlled by the interaction of initiator proteins with genetic elements termed replicators (Jacob et al. 1964). Study of the interaction of purified initiator proteins with *cis*-acting replicators from bacterial chromosomes, bacteriophage, and eukaryotic viruses has led to a general model for the assembly of replication forks at physically defined sites, or origins of replication (Bramhill and Kornberg 1988). In prokaryotes and viruses, the genome commonly consists of a single replicon, with a single replicator controlling initiation of DNA synthesis under all growth conditions. In higher eukaryotes, the genome consists of hundreds to thousands of replicons, and the order of replicon synthesis may vary as a function of growth conditions or cellular differentiation. Since the decision to initiate DNA synthesis is a critical point of control in the cell cycle, there is considerable interest in the structure of eukaryotic replicators and the factors that regulate their selection and activity under various conditions. Although the replicators of mammalian chromosomes and their cognate initiators have yet to be described, the basic unit of DNA synthesis remains the replicon.

REPLICONS AND REPLICON CLUSTERS IN MAMMALIAN CELLS

DNA fiber autoradiography has shown that mammalian DNA replication is usually bidirectional, with equivalent rates of synthesis at each of the two forks in a replication bubble; that initiation begins at multiple, dispersed sites spaced 50–250 kb apart; and that initiation within clusters of contiguous replicons tends to be coordinately controlled (Huberman and Riggs 1968; see also Hand 1978). Thus, at the level of the replicon, DNA synthesis in mammalian cells initiates at a single site, proceeds bidirectionally through the agency of two replication forks, and terminates when forks of neighboring replicons converge.

DNA Replication in Eukaryotic Cells

Localization of replicating DNA in S-phase nuclei by autoradiography or immunohistochemical techniques shows that DNA synthesis occurs in discrete foci, each of which contains several hundred replication forks (Nakamura et al. 1986; Berezney 1991b; Fox et al. 1991; Hassan and Cook 1993). Models for the organization of replication foci suggest that the periodic attachment of replication origins to the nuclear scaffold (or matrix) generates topologically constrained DNA loops that are reeled through fixed sites of synthesis during DNA replication (Pardoll et al. 1980; Nakamura et al. 1986; Berezney 1991a; Cook 1991). In metaphase chromosomes, DNA is also organized in loops that are anchored to a proteinaceous scaffold (Saitoh and Laemmli 1994 and references therein). Although there is a general correlation between the sizes of chromosome loops and replicons (Buongiorno-Nardelli et al. 1982), the precise relationship between replicons, interphase DNA loops, and metaphase chromosome structure is not well understood. To date, no protein involved in the organization of chromosome loops has been linked directly to initiation of DNA synthesis, nor has a functional relationship between scaffold attachment sequences and replication origins been demonstrated.

ASSEMBLY OF REPLICATION COMPLEXES

There is considerable interest in the order of assembly of replication complexes at initiation sites and how the assembly process is regulated during the cell cycle. Immunolocalization of replication factors during the cell cycle suggests that replication complexes are assembled in multiple steps (Kill et al. 1991). Entry into the S phase is accompanied by the redistribution of some replication factors that are constitutively synthesized, such as the DNA polymerase accessory factor proliferating cell nuclear antigen (PCNA) (Bravo and Macdonald-Bravo 1987), to replication foci, whereas other replication factors, such as DNA polymerase-α (Bensch et al. 1982) and cyclin A (Cardoso et al. 1993), are preferentially expressed in G_1 and then recruited to replication sites. In nuclear reconstitution studies with *Xenopus* egg extracts, replication foci are generated during nuclear assembly prior to DNA synthesis (Adachi and Laemmli 1994). Assembly of replication foci in synthetic nuclei requires the single-stranded DNA-binding protein RP-A and inactivation of the mitotic cdc2/cyclin B kinase complex (Adachi and Laemmli 1994). These results suggest that selection of initiation sites occurs postmitotically and is dictated by factors that participate in nuclear reorganization during the transition from mitosis to the G_1 phase.

Assembly of replication foci may not require specific DNA sequences. *Xenopus* egg extracts are able to assemble DNA from any source into synthetic nuclei (Blow and Laskey 1986), which contain replication foci indistinguishable from those of native nuclei (Mills et al. 1989). Moreover, the DNA in synthetic nuclei is duplicated once and only once per nuclear assembly cycle (Blow and Laskey 1988). These results suggest metazoans may not require specific sequence elements to initiate DNA synthesis or to limit replication to once per cell cycle. Nonetheless, localization of DNA methyltransferase within mammalian replication foci requires a distinct targeting sequence (Leonhardt et al. 1992). Thus, as in other replication systems, assembly of mammalian replication complexes occurs through an ordered series of protein-protein interactions. In contrast to other systems, however, the assembly process may be directed by initiation complexes that are not associated with specific DNA sequences.

THE ORDER OF REPLICON SYNTHESIS

There is significant evidence for an ordered, but malleable, program of DNA replication in mammalian cells (see Simon and Cedar, this volume). Transcription appears to have a particularly strong influence on the time during S phase that a sequence is replicated: Genes that are transcriptionally competent tend to be replicated in the first quarter of S phase, whereas those that are not tend to replicate later (Hatton et al. 1988). How might transcription influence replication timing? In early S phase, replication sites coincide with sites of transcription in late G_1 (Hassan et al. 1994), suggesting that, as for some prokaryotic origins, activation of mammalian origins could be linked to the act of transcription (Hassan and Cook 1994). However, the colocalization of transcription and replication sites could be related to the structural organization of transcriptionally active chromatin within the nucleus rather than the process of transcription itself. Alternatively, regulatory transcription factors could participate in the assembly of initiation complexes at both gene promoters and replication origins (for review, see Pederson and Heintz 1994), and this class of DNA-binding proteins could serve to link the two processes in the cell cycle (see van der Vliet, this volume).

THE SEARCH FOR REPLICATION ORIGINS IN MAMMALIAN CELLS

The genetic elements involved in initiation of DNA synthesis are well described in only one eukaryote—the yeast *Saccharomyces cerevisiae.*

Minimal replicators from *S. cerevisiae* contain at least two functional domains: Domain A encompasses a core consensus sequence (the ACS) that is necessary, but not sufficient, for origin activity, and domain B harbors heterogeneous accessory sequences that enhance the efficiency of origin utilization (see Newlon, this volume). The fact that heterogeneous accessory elements act in concert with the yeast origin core suggests that yeast replicators resemble gene promoters (Heintz et al. 1992), a notion reinforced by the observation that transcription factors stimulate yeast origin activity (for review, see Pederson and Heintz 1994).

The time of activation during S phase and the frequency of origin utilization in yeast are subject to chromosomal context (Fangman and Brewer 1991), suggesting that long-range chromatin determinants or other factors (i.e., transcription) influence the distribution or activity of yeast initiator proteins. Indeed, a strong candidate for the *S. cerevisiae* initiator protein, the origin recognition complex (ORC), functions in both DNA replication (Bell and Stillman 1992) and the repression of transcription (Foss et al. 1993). ORC is bound to the yeast origin *ARS1* throughout most of the cell cycle (Diffley and Cocker 1992), and proteins that interact with ORC in a cell-cycle-dependent manner appear to control origin activation during entry into S phase (Diffley et al. 1994; Dowell et al. 1994). Presumably, the proteins that interact with ORC to achieve repression of transcription differ from those that interact with ORC during initiation of DNA replication.

Given the high degree of conservation of replication enzymes and the kinase cascades that control entry into the S phase in eukaryotes, it would be surprising if animal cells did not contain homologs of ORC. Nonetheless, replication origins in mammalian cells clearly differ from those in *S. cerevisiae*. In *S. cerevisiae*, replication of episomal plasmids requires specific DNA sequences, thereby allowing dissection of the *cis*-acting elements involved in replicator function. The identification of mammalian replicators by plasmid replication assays has proven far less successful. Regions of the c-*myc* promoter (McWhinney and Leffak 1990), the adenosine deaminase gene region (Virta-Pearlman et al. 1993), hsp70 gene promoter (Taira et al. 1994), and several anonymous sequences (Frappier and Zannis-Hadjopoulos 1987; Masukata et al. 1993; Nielson et al. 1994) have been reported to support the episomal replication of recombinant plasmids. In several instances, the sequences that promote plasmid replication have been shown to serve as initiation sites in the chromosome (see DePamphilis, this volume). However, significant variations in the results of plasmid transfection experiments have been observed in different laboratories (Burhans et al. 1990; Caddle and

Calos 1992; Matsukata et al. 1993), suggesting that the assay may not accurately detect *cis*-acting sequences of chromosomal replicators. Study of initiation activity with an assay based on the nuclear retention of plasmids by the Epstein-Barr virus EBNA1 protein indicates that size, and not primary sequence composition, is the most critical factor affecting the efficiency of plasmid replication in animal cells (Krysan et al. 1989). Additional studies in the EBNA1 system indicate that initiation occurs at random sites in plasmid DNA (Krysan and Calos 1991) and is precluded only from transcribed regions (Haase et al. 1994).

ARS activity in plasmids may reflect the relative propensity of different DNA sequences to unwind under superhelical stress. When supercoiled plasmids are incubated with DNA polymerase-α:primase, RP-A, T antigen, and gyrase in vitro, initiation is observed at the c-*myc* promoter, the yeast origin *ARS1*, and, to a lesser extent, the DHFR *oriβ* region (Ishimi et al. 1994). A survey of human DNA for the ability to support priming of DNA synthesis by DNA polymerase-α:primase in the presence of RP-A in vitro showed that site-specific synthesis occurs preferentially at sequences containing the dinucleotide repeat dA:dT (Pack and Tsurimoto 1994). Since poly(dA:dT) repeats have a low free energy for unwinding, these sequences tend to become single-stranded in supercoiled plasmids (Caddle et al. 1990b; Pack and Tsurimoto 1994), a property that accounts for their preferential use as in vitro initiation sites. Although DNA unwinding elements (DUEs) are important components of replication origins (see DePamphilis, this volume), further work is required before a relationship between sequences that promote plasmid DNA replication and those that function as replicators in the chromosome can be established.

INITIATION SITES IN MAMMALIAN CHROMOSOMES

Without a definitive genetic assay, mapping of initiation events to specific sites in mammalian chromosomes has been largely limited to methods that are based on the conserved features of replication forks. Replication forks in animal cells have the same organization as those in other organisms: DNA synthesis is semi-discontinuous, with one template strand duplicated by a highly processive polymerase complex, and the other copied by a discontinuous mechanism through the agency of Okazaki fragments (see, e.g., Waga and Stillman 1994). Since mammalian DNA replication is semi-discontinuous and bidirectional, origin-mapping methods are based on assays that assess the template specificity of leading- and lagging-strand synthesis, polarity of replication fork

movement, stability of DNA in replication bubbles, size of nascent DNA strands, or electrophoretic migration of replication forks and bubbles in two-dimensional (2D) gels (for review, see Vassilev and DePamphilis 1992). Similarly, labeling of DNA during entry into the S phase has been used to isolate initiation sites. However, as has been noted previously (Stillman 1994), mapping of initiation sites is not synonymous with mapping the *cis*-acting elements of replicators. For example, mapping of transcription start sites would not necessarily identify *cis*-acting enhancer elements that act at a distance to influence gene expression.

Using a variety of techniques, origin-mapping experiments have located initiation sites near the c-*myc* promoter region (Vassilev and Johnson 1990), two different regions of the murine adenosine deaminase gene locus (Carroll et al. 1993; Virta-Pearlman et al. 1993), the immunoglobulin heavy-chain enhancer (Ariizumi et al. 1993), the rhodopsin gene locus (Gale et al. 1992), the nontranscribed portion of the rDNA repeats (Little et al. 1993), the RPS14 gene (Tasheva and Roufa 1994a), the lamin B2 gene (Giacca et al. 1994), the hsp70 gene (Taira et al. 1994), the human β-globin locus (Kitsberg et al. 1993), and the Chinese hamster dihydrofolate reductase (DHFR) domain (see below). These initiation sites show no obvious spatial relationship to transcription units, and features of these sequences that contribute to their use as initiation sites are difficult to discern. Since regulatory transcription factors have been shown to act as accessory factors in the initiation of viral and yeast DNA replication (for review, see Pederson and Heintz 1994), the distribution of transcription factor binding sites is potentially interesting. However, the consensus sequences for transcription factor binding sites tend to be short and tolerate a number of substitutions, and therefore these sequences tend to be widely distributed in genomic DNA (Dobbs et al. 1994). Binding sites for the sequence-specific single-stranded DNA-binding protein Pur are found in gene promoters and mammalian initiation sites at a higher frequency than expected by chance (Bergemann and Johnson 1992; Dobbs et al. 1994). A Pur-binding site juxtaposed to the JC virus core replication origin has been shown to be important for both viral DNA replication (Chang et al. 1994) and early gene transcription (Chen et al. 1995), suggesting that Pur could also play a role in mammalian replication. Although comparison of six initiation sites has identified a degenerate consensus sequence (Dobbs et al. 1994), the consensus has not been shown to have a function analogous to the ACS of *S. cerevisiae* replicators.

Given the lack of a definitive assay, demonstration that specific sequences or DNA-binding proteins are involved in initiation in mam-

malian cells is certain to be difficult. An additional factor that confounds the identification of replicators and initiators in mammalian cells is the apparent decentralization of initiation events, a phenomenon that has been documented most thoroughly in the DHFR domain of Chinese hamster cells.

THE CHINESE HAMSTER DHFR AMPLICON

During the past decade, several laboratories have studied replication of the DHFR domain of Chinese hamster ovary (CHO) cells in an effort to identify the sequences that regulate initiation of DNA synthesis in mammalian cells. Since the CHO DHFR system has been examined with every available replication assay, it provides a good perspective of the search for mammalian replicators. In addition, recent studies link the promoter of the DHFR gene to initiation of DNA synthesis, an observation that reinforces the suggestion that DNA replication and gene expression are intimately intertwined processes.

Study of the Chinese hamster DHFR domain began with the observation that the mitotic chromosomes of methotrexate-resistant CHO cells contain large regions that display a monotonous Giemsa staining pattern. In the methotrexate-resistant cell strain CHOC 400, homogeneously staining regions (HSRs) were shown to encompass about 1000 copies of the gene that encodes DHFR, the enzyme inhibited by methotrexate (Milbrandt et al. 1981). Molecular cloning showed that each amplified DHFR gene of CHOC 400 cells is embedded in a tandemly repeated unit (or amplicon) of about 250 kb (Looney and Hamlin 1987). Each CHOC 400 amplicon contains at least three transcription units: the 25-kb DHFR gene (Milbrandt et al. 1983), the 65-kb Rep3 gene that encodes a *MutS* homolog (Mitchell et al. 1986), and the 34-kb 2BE2121 gene that encodes a protein of unknown function (Foreman and Hamlin 1989). The organization of the CHOC 400 DHFR amplicon is shown in Figure 1.

Retroactive labeling of mitotic chromosomes showed that replication of the HSRs begins at the onset of the CHOC 400 S phase (Milbrandt et al. 1981), suggesting that each DHFR amplicon contains at least one replication origin that is activated early in the S phase. Pulse-labeling studies in synchronized CHOC 400 cells identified a series of amplified restriction fragments that begin replication early in the S phase (Heintz and Hamlin 1982). Cells synchronized with cytosine arabinoside, aphidicolin, or hydroxyurea showed the same pattern of fragment labeling in early S (Heintz and Hamlin 1982, 1983), suggesting metabolic inhibitors per se do not influence the selection of initiation sites. Cloning

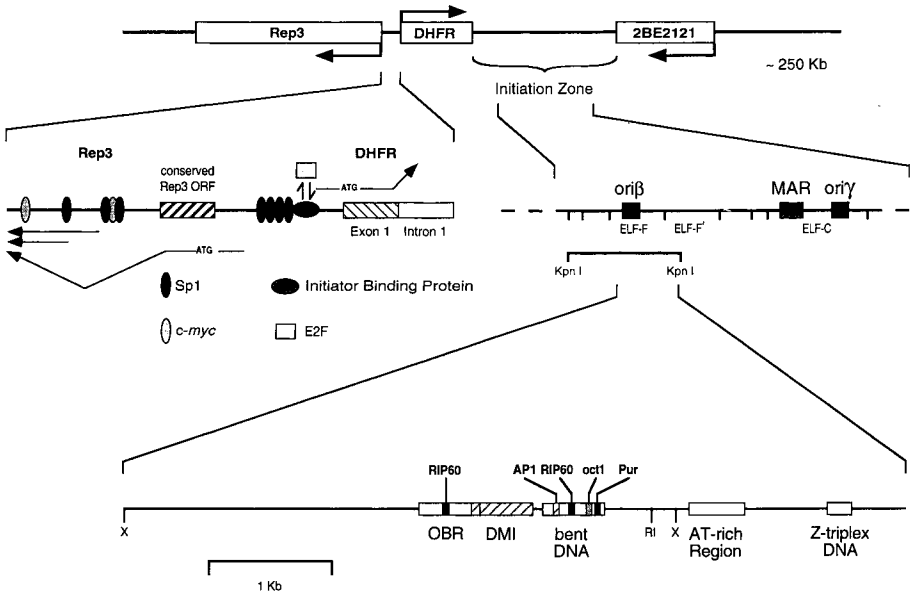

Figure 1 Organization of the Chinese hamster DHFR amplicon. The direction of transcription through the DHFR, Rep3, and 2BE2121 transcription units (*boxes*) within the 250-kb DHFR amplicon is indicated by arrows. Genomic footprinting of protein-DNA interactions within the bidirectional DHFR/rep3 promoter region shows that E2F regulates induction of DHFR transcription during traversal of the G_1 phase (*right* panel). Early-labeled *Eco*RI fragments F, F′, and C from the intergenic initiation zone are shown relative to *ori*β, *ori*γ, and a matrix attachment region (MAR). Sequence elements near the origin of bidirectional DNA replication (OBR)/*ori*β include a densely methylated island (DMI), a region of stably bent DNA that binds multiple protein factors, a 450-bp AT-rich region, and a Z-triplex DNA motif. Binding sites for RIP60, AP1, Oct-1, and Pur within these sequence motifs are shown. In the lower panel, X indicates the boundaries of the 4.3-kb *Xba*I fragment discussed in the text.

and mapping showed the early-labeled restriction fragments (ELFs) are located between the convergently transcribed DHFR and 2BE2121 transcription units (Fig. 1) (Heintz et al. 1983).

A number of approaches have been used in an effort to more precisely map initiation events in the intergenic ELF region. Hybridization of DNA that had been pulse-labeled with radiolabeled thymidine during various intervals of the CHOC 400 S phase to immobilized plasmids suggested initiation occurs with high frequency in a 4.3-kb *Xba*I fragment that maps largely within ELF-F (Fig. 1) (Burhans et al. 1986a). Preferential labeling of this fragment was also observed when cytosine

arabinoside was used to stall replication forks as cells entered S phase (Burhans et al. 1986b), or UV-induced cross-linking of the template with psoralen was used to inhibit the movement of replication forks away from initiation sites (Anachkova and Hamlin 1989). Using an in-gel-renaturation technique to enrich for signals from amplified restriction fragments, preferential labeling during the onset of the S phase was shown to occur within two short regions (termed *oriβ* and *oriγ*) that are separated by approximately 20 kb (Leu and Hamlin 1989). *oriβ* maps within the 4.3-kb *Xba*I fragment of ELF-F, and *oriγ* maps within ELF-C (Fig. 1).

More precise mapping methods based on the template specificity of leading- and lagging-strand synthesis also indicate that initiation occurs with high frequency at *oriβ*. Since the two forks within a replication bubble travel in opposite directions, and chromosomal DNA synthesis is semi-discontinuous, lagging- and leading-strand synthesis must switch template strands at the center of the replication bubble where DNA synthesis begins. Template switch points that physically demarcate leading- and lagging-strand initiation sites have been operationally defined as origins of bidirectional DNA replication (OBRs) (Vassilev and DePamphilis 1992). Using a nuclear run-on replication assay (Heintz and Stillman 1988), Okazaki fragments from CHOC 400 cells were labeled with [^{32}P]dNTPs in vitro and hybridized to strand-specific M13 clones from the DHFR ELF region. Quantitation of the strand-specific hybridization signals showed an OBR is located within a 450-bp region of the 4.3-kb early-replicating *Xba*I fragment that contains *oriβ* (Burhans et al. 1990).

Mapping of an OBR to *oriβ* with Okazaki fragments is supported by studies on the template specificity of leading-strand intermediates. When cells are treated with the protein synthesis inhibitor emetine, Okazaki fragment synthesis is rapidly inhibited, leading to the preferential accumulation of leading-strand replication intermediates (Burhans et al. 1991). Mapping of the strand specificity of leading-strand intermediates showed that DNA replication begins preferentially at two sites within the DHFR initiation region, one of which maps at the OBR/*oriβ*, and the second near *oriγ* (Handeli et al. 1989). A significant finding from the mapping of leading-strand intermediates is that the initiation sites that are used in CHOC 400 cells also are used in CHO cells that contain only two copies of the DHFR domain, a finding confirmed with PCR-based assays that measure the amount and length of nascent DNA strands relative to specific chromosomal positions (Vassilev et al. 1990; Tasheva and Roufa 1994b; M. Giacco, pers. comm.).

Although there is convincing evidence that initiation of DNA synthesis occurs with high frequency at the *ori*β/OBR located in ELF-F, the conclusion that initiation occurs at a limited number of preferred sites in the DHFR origin region is not supported by the analysis of cellular replication intermediates with 2D gels. With neutral-neutral 2D gels, replication bubbles have been detected in every restriction fragment spanning the 55 kb of DNA that lies between the DHFR and 2BE2121 transcription units (Vaughn et al. 1990; Dijkwel and Hamlin 1992; Dijkwel et al. 1994). Neutral-alkaline 2D gel analysis indicates replication forks travel in both directions through many fragments in the initiation zone (Vaughn et al. 1990; Dijkwel et al. 1994), a finding that also indicates initiation occurs at many dispersed sites. Although it is difficult to compare the relative frequency of initiation events at different sites by 2D gels, taken together, the 2D gel results indicate initiation occurs at many sites, perhaps even randomly, throughout the entire DHFR initiation zone (Fig. 1).

There is strong evidence that the 2D gel patterns from CHO cells reflect true chromosomal replication intermediates. The migration patterns of replication intermediates from yeast and CHO cells are indistinguishable (J.A. Huberman and J.L. Hamlin, pers. comm.), replication intermediates are limited to S phase (Dijkwel and Hamlin 1992; Dijkwel et al. 1994), and inhibitors of DNA synthesis alter the migration of replication intermediates in 2D gels in a predictable fashion (Levenson and Hamlin 1993). 2D gel analysis of replication intermediates from the ribosomal DNA locus in human cells also suggests initiation occurs at multiple sites within nontranscribed spacer regions (Little et al. 1993). Models that have been proposed to account for the apparent discrepancies between 2D gel and other mapping techniques have not been supported by experimental evidence. Resolution of this apparent paradox (i.e., random versus site-specific initiation) will require the examination of other initiation regions by multiple techniques, and a stronger understanding of the relationship between 2D gel patterns and the transient replication intermediates detected by labeling studies or PCR-based techniques.

GENETIC ASSAYS FOR DHFR ORIGIN ACTIVITY

Cloning of the DHFR initiation region led to transfection experiments to identify autonomously replicating sequences analogous to yeast ARS elements. Because these experiments have been largely unsuccessful, they are not widely reported in the literature (see, e.g., Burhans et al. 1990).

Nonetheless, plasmids carrying fragments that span 40 kb of the DHFR initiation zone (including the OBR) have been tested for autonomous replication after transfection by several methods into CHO, CHOC 400, HeLa, 293, and COS-7 cells, all without success (N.H. Heintz, unpubl.; J.L. Hamlin, pers. comm.). Recently, the DHFR OBR was reported to support autonomous plasmid replication in vivo and in vitro (Zannis-Hadjopoulos et al. 1995). This latter study, however, did not directly compare ARS activity of the DHFR OBR to size-matched controls. Using the neutral-neutral 2D gel assay, Caddle and Calos (1992) tested the ability of a 14-kb *Kpn*I genomic fragment that encompasses the OBR to support site-specific initiation in plasmids that are retained in the nucleus by the viral protein EBNA1. In these experiments, 2D gel analysis showed that initiation occurred at random throughout the DHFR sequences, with no detectable preference for the OBR. Unfortunately, PCR or strand-specific mapping assays were not compared to the 2D gel results in this study.

In *Drosophila melanogaster*, developmentally regulated origins of replication known as amplification control elements (ACE) mediate the amplification of flanking sequences when transferred to new chromosomal positions (Orr-Weaver et al. 1989). Using a plasmid transfection assay, Stolzenburg et al. (1994) have reported that the DHFR OBR promotes the amplification of plasmid DNA concatemers in long-term transfection experiments. To determine if the DHFR origin region enhances the rate of chromosomal gene amplification in a manner similar to *Drosophila* ACEs, the 14-kb *Kpn*I fragment that encompasses the OBR was linked to a DHFR minigene and transfected into *dhfr*$^-$ CHO-DUKX cells (Brinton 1991). After selection for DHFR enzyme activity, individual cell clones were selected for methotrexate resistance. Amplification of the integrated DHFR minigene was found to be dependent on chromosomal position; inclusion of the DHFR initiation region did not mediate position-independent gene amplification, nor did these sequences influence the rate at which the DHFR minigene was amplified (Brinton 1991). Although heterologous genetic assays have failed to detect site-specific initiation or amplification activity in the 14-kb *Kpn*I fragment, these sequences must have distinctive properties in the chromosome, for they are the only portion of the DHFR amplicon that is entirely refractory to UV-induced DNA repair (Ho et al. 1989).

FEATURES OF THE DHFR OBR

Although a *cis*-acting replicator for the DHFR gene has not been described, every origin-mapping study (including those utilizing 2D gels)

indicates that initiation of replication occurs with high frequency within the 4.3-kb *Xba*I fragment that contains *ori*β/OBR. A 6-kb region surrounding *ori*β/OBR has been sequenced (Caddle et al. 1990b) and examined for DNA sequences with unusual structure, DNA unwinding elements, and protein-binding sites. Located near the 3′ end of the sequence is a 180-bp stretch of simple alternating dinucleotide repeats that forms left-handed Z and triple-stranded (or triplex) DNA when subjected to superhelical stress in vitro (Bianchi et al. 1990; Caddle et al. 1990b). Primer extension studies show that DNA polymerases traverse this element more readily in the Z-to-triplex than triplex-to-Z DNA direction in vitro (Brinton et al. 1991), suggesting that the orientation of this sequence may influence the rate of replication fork travel in vivo. Although orientation and position of the Z-triplex motif has dramatic effects on the rate of SV40-origin-dependent plasmid replication in COS-7 cells (Brinton et al. 1991), 2D gel analysis showed the Z-triplex sequence is not an absolute fork barrier, but rather causes the accumulation of late-replication intermediates in plasmids. Neutral-neutral 2D gel analysis has not detected a replication fork barrier at the Z triplex motif in situ (Vaughn et al. 1990; Dijkwel and Hamlin 1992; Dijkwel et al. 1994), and the function of this motif in the genome, if any, is unknown.

The DHFR initiation region contains several tracts of AT-rich sequences, including a prominent 450-bp segment that is 75% AT and contains multiple near-matches to the *S. cerevisiae* ACS (Fig. 1). A hierarchy of DNA unwinding elements in the DHFR initiation region was determined by digesting a panel of supercoiled plasmids with mung bean nuclease (MBN) as described by Kowalski and Eddy (1989). In the MBN assay, the DNA that was most readily unwound under superhelical stress is a simple alternating dA:dT repeat of 46 bp that maps about 1000 bp 3′ to the OBR (Caddle et al. 1990b). As discussed earlier, poly(dA:dT) sequences support DNA unwinding and priming of DNA synthesis in vitro (Caddle et al. 1990b; Pack and Tsurimoto 1994). DNA unwinding was not detected in the 450-bp AT-rich region, nor was the OBR susceptible to nuclease scission, even when isolated from flanking sequences (Caddle et al. 1990b). Located approximately 700 bp downstream from the OBR is a region of stably bent DNA that binds multiple protein factors, including the transcription factors AP1 and Oct-1 (Held et al. 1992), the DNA-binding protein RIP60 (Dailey et al. 1990), and the single-stranded DNA-binding protein Pur (Bergemann and Johnson 1992). The bent DNA AP1 site binds c-jun/c-jun and c-fos/c-jun heterodimers in vitro (Held et al. 1992), a result of interest since c-fos/c-jun heterodimers have been shown to be potent enhancers of SV40 and polyoma core

origin activity in transient replication assays (Guo and DePamphilis 1992). Recently, the DHFR bent DNA motif was shown to increase the activity of the polyoma core origin 20-fold in transient replication assays, and the level of replication increased to 50-fold when the test plasmid was cotransfected with expression vectors for c-fos and c-jun (K. Murakami et al., in prep.).

Footprinting of the bent DNA region with HeLa cell nuclear extract in vitro identified a 60-kD DNA-binding protein called RIP60 that binds to an ATT-repeat motif 20 bp 3′ to the bent DNA AP1 site (Fig. 1) (Dailey et al. 1990). RIP60 bends DNA toward the major groove about 82° (Caddle et al. 1990a; M. Soultanakis and N. H. Heintz, unpubl.) and, when bound to the native bent DNA motif, counteracts the direction of DNA-directed bending. Further work showed that RIP60 binds to a second site within the 450 bp of the 4.3-kb *Xba*I fragment that includes the OBR (P. Held and N. H. Heintz, unpubl.). Dimers of RIP60 bound to both sites mediate the formation of a 710-bp twisted DNA loop in vitro (Mastrangelo et al. 1993). Thus, DNA looping by RIP60 links an AP1-dependent replication enhancer to the OBR in vitro. Recently, the sequences between the two RIP60-binding sites were shown to encompass a large densely methylated island (DMI) in which virtually all dC residues are methylated (Tasheva and Roufa 1994b). Methylation of the DMI is prominent in cycling cells, is lost in quiescent cells, and reappears within 48 hours after quiescent cells are stimulated to reenter the cell cycle. A second DMI has been mapped near an initiation site associated with the RPS14 gene (Tasheva and Roufa 1994a). These reports suggest that growth-regulated changes in DNA methylation may influence initiation of DNA replication, and they raise the possibility that the failure of transfection assays to identify mammalian origin elements may be due to lack of proper methylation of the DNA template, a post-synthetic modification that is absent in yeast.

REPLICATION OF THE DHFR AMPLICON IN NUCLEI

Cell fusion experiments show that S-phase mammalian cells contain a diffusible signal that induces DNA synthesis in G_1 nuclei. In an attempt to reproduce this finding in vitro, a nuclear run-on replication assay was developed for CHOC 400 cells (Heintz and Stillman 1988). Incubation of nuclei with cytosol from cells staged at various points in the cell cycle showed that S-phase extracts support higher rates of DNA synthesis in S-phase nuclei than do G_1 extracts, and that G_1 extracts do not contain a dominant inhibitor of DNA replication. However, under these conditions,

S-phase extracts do not induce initiation of DNA synthesis in CHOC 400 nuclei isolated from G_1 cells.

In contrast, *Xenopus* egg extracts that support nuclear assembly and DNA replication in vitro are able to induce DNA synthesis in nuclei isolated from CHOC 400 cells arrested in G_1 (Gilbert et al. 1993). Synthesis begins in the DHFR initiation zone, suggesting that the protein factors and nuclear structure that dictate selection of initiation sites reside in CHOC 400 G_1 nuclei, and that *Xenopus* extracts can activate mammalian initiation complexes (Gilbert et al. 1995). Interestingly, induction of DNA synthesis is sensitive to the protein kinase inhibitor 6-DMAP (Gilbert et al. 1995), a finding that indicates kinases in the *Xenopus* extract are able to phosphorylate factors in the extract or in the G_1 nucleus required for entry into the S phase. Fractionation of the *Xenopus* extract may lead to the identification of kinase activities required to activate mammalian replication complexes.

LINKS BETWEEN TRANSCRIPTION AND REPLICATION IN THE DHFR AMPLICON

Density shift (Caddle and Heintz 1990) and 2D gel analysis of replication intermediates (Vaughn et al. 1990; Dijkwel and Hamlin 1992) show that initiation occurs in only one of ten DHFR amplicons during the typical cell cycle. Since the DHFR amplicons share the same primary sequence, failure to initiate at every amplicon could be due to several factors: (1) The initiation region of each amplicon may not be associated with the nuclear matrix, a notion supported by preliminary experimental evidence (Dijkwel and Hamlin 1988); (2) there may be insufficient amounts of initiation proteins and replication factors for the assembly of replication complexes at all amplicons; and (3) initiation may be linked to transcription of the DHFR gene, and each copy of the DHFR gene may not be transcribed during every cell cycle.

A link between transcription of the DHFR gene and initiation is strengthened by the observation that chromosomal deletions that remove the DHFR promoter, but not the body of the DHFR gene or the 3′ origin region, delay or abolish initiation in the early-replicating region (V. Levenson et al., pers. comm.). Genomic footprinting of the bidirectional DHFR/Rep3 promoter region during the cell cycle shows that in G_1 the DHFR promoter is bound by a basal transcription complex that includes the transcription factor Sp1 and a protein bound at the transcription start site, and that elevated expression of DHFR during traversal of G_1 is correlated with occupancy of an E2F transcription factor binding site (see

Fig. 1) (J. Wells et al., in prep.). The promoter footprinting studies show that all 1000 copies of the DHFR promoter in CHOC 400 cells are occupied by a basal transcription complex, suggesting that failure to initiate replication in each amplicon during every cell cycle is not due to differences in access to general or regulatory transcription factors.

Evidence from another system suggests that gene promoters may influence replication by mechanisms other than inducing transcription per se. Deletion of an 8-kb segment of the human β-globin domain alters the template specificity of leading-strand DNA synthesis in the globin domain from a divergent, bidirectional pattern to a unidirectional pattern (Kitsberg et al. 1993), suggesting that the deletion has removed one or more elements of a replicator required for initiation of bidirectional replication at a specific site. The globin deletion alters the replication pattern whether or not the locus is transcribed, suggesting that in addition to regulating transcription, promoter sequences may have additional functions, such as promoting the association of the globin domain with the nuclear matrix. Other deletions in the globin locus, far removed from the initiation site, change the replication timing and long-range chromatin structure of this region, alterations that also influence globin gene expression (Forrester et al. 1990).

SUMMARY

Given the lack of a reliable genetic assay; the fact that specific sequence elements are not required for nuclear assembly, initiation of DNA synthesis, or limiting replication to once per cell cycle in *Xenopus* egg extracts; and the heterogeneity of sequences that appear to function as initiation sites in mammalian cells, a perplexing picture of mammalian replicators has emerged. How might initiation sites be selected in animal cells? First, the deposition of initiation complexes on DNA may be determined by a combination of primary sequence elements, chromatin organization, and nuclear structure. Study of the flanking sequences that affect the time and frequency of activation of replication origins in yeast may provide important clues about the determinants that influence the selection of mammalian initiation sites. Second, fixed *cis*-acting replicator sequences could act at a distance (perhaps by DNA looping) to inspire initiation at multiple sites. If mammalian replicators resemble gene promoters, *cis*-acting replicator elements could be dispersed over thousands of kilobase pairs and overlap with those elements required for regulation of transcription and chromatin organization within the same replicon. This possibility is attractive, for it suggests that factors which

influence nuclear structure could account for the strong link between replication and transcription control. Third, as proposed by Benbow and colleagues (Benbow et al. 1992), DNA unwinding could commence at specific sites but be uncoupled from the assembly of replication forks, leading to a decentralized initiation pattern. Given the complexity of development and differentiation in mammals, it is not surprising that there are significant differences between replication origins in mammalian cells and yeast. The task at hand is to understand the significance of these differences in the context of eukaryotic evolution.

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REFERENCES

Adachi, Y. and U.K. Laemmli. 1994. Study of the cell cycle-dependent assembly of the pre-replication centers in *Xenopus* egg extracts. *EMBO J.* **13:** 4153–4164.

Anachkova, B. and J.L. Hamlin. 1989. Replication in the amplified dihydrofolate reductase domain in CHO cells may initiate at two distinct sites, one of which is a repetitive sequence element. *Mol. Cell. Biol.* **9:** 532–540.

Ariizumi, K., Z. Wang, and P.W. Tucker. 1993. Immunoglobulin heavy chain enhancer is located near or in an initiation zone of chromosomal DNA replication. *Proc. Natl. Acad. Sci.* **90:** 3695–3699.

Bell, S.P. and B. Stillman. 1992. ATP-dependent recognition of eukaryotic origins of replication by a multi-protein complex. *Nature* **357:** 128–134.

Benbow, R.M., J. Zhao, and D.D. Larson. 1992. On the nature of origins of DNA replication in eukaryotes. *BioEssays* **14:** 661–670.

Bensch, K.G., S. Tanaka, S.-Z. Hu, T.S.-F. Wang, and D. Korn. 1982. Intracellular localization of human polymerase α with monoclonal antibodies. *J. Biol. Chem.* **257:** 8391–8396.

Berezney, R. 1991a. The nuclear matrix: A heuristic model for investigating genomic organization and function in the cell nucleus. *J. Cell. Biochem.* **47:** 109–123.

———. 1991b. Visualizing DNA replication sites in the cell nucleus. *Semin. Cell Biol.* **2:** 103–115.

Bergemann, A.D. and E.M. Johnson. 1992. The HeLa Pur factor binds single-stranded DNA at a specific element conserved in gene flanking regions and origins of DNA replication. *Mol. Cell. Biol.* **12:** 1257–1265.

Bianchi, A., R.D. Wells, N.H. Heintz, and M.S. Caddle. 1990. Sequences near the origin of replication at the *dhfr* locus of Chinese hamster ovary cells adopt left-handed Z-DNA and triplex structures. *J. Biol. Chem.* **265:** 21789–21796.

Blow, J.J. and R.A. Laskey. 1986. Initiation of DNA replication in nuclei and purified DNA by a cell-free extract of *Xenopus* eggs. *Cell* **47:** 577–587.

———. 1988. A role for the nuclear envelope in controlling DNA replication within the

cell cycle. *Nature* **332:** 546–548.

Bramhill, D. and A. Kornberg. 1988. A model for initiation at origins of DNA replication. *Cell* **54:** 915–918.

Bravo, R. and H. Macdonald-Bravo. 1987. Existence of two populations of cyclin/proliferating cell nuclear antigen during the cell cycle: Association with DNA replication sites. *J. Cell Biol.* **105:** 1549–1554.

Brinton, B. 1991. "Effect of chromosomal position on the mode and frequency of gene amplification in mammalian cells." Ph.D. thesis, University of Vermont, Burlington.

Brinton, B., M.S. Caddle, and N.H. Heintz. 1991. Position and orientation-specific effects of a Z-triplex DNA motif on episomal DNA replication in COS-7 cells. *J. Biol. Chem.* **266:** 5153–5161.

Buongiorno-Nardelli, M., M.G. Micheli, M.T. Carri, and M. Marilley. 1982. A relationship between replicon size and supercoiled loop domains in the eukaryotic genome. *Nature* **298:** 100–102.

Burhans, W.C., J.E. Selegue, and N.H. Heintz. 1986a. Replication intermediates formed during initiation of DNA synthesis in methotrexate-resistant CHOC 400 cells are enriched for sequences from a specific, amplified restriction fragment. *Biochemistry* **25:** 441–449.

———. 1986b. Isolation of the origin of replication associated with the amplified Chinese hamster ovary dihydrofolate reductase domain. *Proc. Natl. Acad. Sci.* **83:** 7790–7794.

Burhans, W.C., L.T. Vassilev, M.S. Caddle, N.H. Heintz, and M.L. DePamphilis. 1990. Identification of an origin of bidirectional DNA replication in mammalian chromosomes. *Cell* **62:** 955–965.

Burhans, W.C., L.T. Vassilev, J. Wu, J.M. Sogo, F.S. Nallaseth, and M.L. DePamphilis. 1991. Emetine allows identification of origins of mammalian DNA replication by imbalanced DNA synthesis, not through conservative nucleosome segregation. *EMBO J.* **10:** 4351–4360.

Caddle, M.S. and M.P. Calos. 1992. Analysis of the autonomous replication behavior in human cells of the DHFR putative chromosomal origin of replication. *Nucleic Acids Res.* **20:** 5971–5978.

Caddle, M.S. and N.H. Heintz. 1990. The replication timing of the amplified dihydrofolate reductase genes in the Chinese hamster ovary cell line CHOC 400. *Biochem. Biophys. Res. Commun.* **170:** 134–139.

Caddle, M.S., L. Dailey, and N.H. Heintz. 1990a. RIP60, a mammalian origin-binding protein, enhances DNA bending near the dihydrofolate reductase origin of replication. *Mol. Cell. Biol.* **10:** 6236–6243.

Caddle, M.S., R.H. Lussier, and N.H. Heintz. 1990b. Intramolecular DNA triplexes, bent DNA and DNA unwinding elements in the initiation region of an amplified dihydrofolate reductase replicon. *J. Mol. Biol.* **211:** 19–33.

Cardoso, M.C., H. Leonhardt, and B. Nidal-Ginard. 1993. Reversal of terminal differentiation and control of DNA replication: Cyclin A and cdk2 specifically localize at subnuclear sites of DNA replication. *Cell* **74:** 979–992.

Carroll, S.M., M.L. DeRose, J.L. Kolman, G.H. Nonet, R.E. Kelly, and G.M. Wahl. 1993. Localization of a bidirectional DNA replication origin in the wild type and in episomally amplified murine ADA loci. *Mol. Cell. Biol.* **13:** 2971–2981.

Chang, C.-F., H. Tada, and K. Khalili. 1994. The role of a pentanucleotide repeat sequence, AGGGAAGGGA, in the regulation of JC virus DNA replication.

Gene **148:** 309–314.

Chen, N.N., C.-F. Chang, G.L. Gallia, D.A. Kerr, E.M. Johnson, C.P. Krachmarov, S.M. Barr, R.J. Frisque, B. Bollage, and K. Khalili. 1995. Cooperative action of the cellular proteins YB-1 and Purα with the tumor antigen of the human JC polyomavirus determines their interaction with the viral lytic control element. *Proc. Natl. Acad. Sci.* **92:** 1087–1091.

Cook, P.R. 1991. The nucleoskeleton and the topology of replication. *Cell* **66:** 627–635.

Dailey, L., M.S. Caddle, N. Heintz, and N.H. Heintz. 1990. Purification of RIP60 and RIP100, mammalian proteins with origin specific DNA binding and ATP-dependent DNA helicase activities. *Mol. Cell. Biol.* **10:** 6225–6235.

Diffley, J.F.X. and J.H. Cocker. 1992. Protein-DNA interactions at a yeast replication origin. *Nature* **357:** 169–172.

Diffley, J.F.X., J.H. Cocker, S.J. Dowell, and A. Rowley. 1994. Two steps in the assembly of complexes at yeast replication origins in vivo. *Cell* **78:** 303–316.

Dijkwel, P.A. and J.L. Hamlin. 1988. Matrix attachment regions are positioned near replication initiation sites, genes, and an interamplicon junction in the amplified dihydrofolate reductase domain of Chinese hamster ovary cells. *Mol. Cell. Biol.* **8:** 5398–5409.

———. 1992. Initiation of DNA replication in the DHFR locus is confined to the early S-period in CHO cells synchronized with the plant amino acid mimosine. *Mol. Cell. Biol.* **12:** 3715–3722.

Dijkwel, P.A., J.P. Vaughn, and J. L. Hamlin. 1994. Replication initiation sites are distributed widely in the amplified CHO dihydrofolate reductase domain. *Nucleic Acids Res.* **22:** 4989–4996.

Dobbs, D.L., W.-L. Shaiu, and R.M. Benbow. 1994. Modular sequence elements associated with origin regions in eukaryotic chromosomal DNA. *Nucleic Acids Res.* **22:** 2479–2489.

Dowell, S.J., P. Romanowski, and J. F. Diffley. 1994. Interaction of Dbf4, the Cdc7 protein kinase regulatory subunit, with yeast replication origins in vivo. *Science* **265:** 1243–1246.

Fangman, W.L. and B.S. Brewer. 1991. Activation of replication origins within yeast chromosomes. *Annu. Rev. Cell Biol.* **7:** 375–402.

Foreman, P.K. and J.L. Hamlin. 1989. Identification and characterization of a gene that is coamplified with dihydrofolate reductase in a methotrexate-resistant CHO cell line. *Mol. Cell. Biol.* **9:** 1137–1147.

Forrester, W.C., E. Epner, M.C. Driscoll, T. Enver, M. Brice, T. Papayannopoulou, and M. Groudine. 1990. A deletion of the human β-globin locus activation region causes a major alteration in chromatin structure and replication across the entire β-globin region. *Genes Dev.* **4:** 1637–1649.

Foss, M., F.J. McNally, P. Laurenson, and J. Rine. 1993. Origin recognition complex (ORC) in transcriptional silencing and DNA replication in *S. cerevisiae*. *Science* **262:** 1838–1844.

Fox, M.H., D.J. Arndt-Jovin, T.M. Jovin, P.H. Baumann, and M. Robert-Nicoud. 1991. Spatial and temporal distribution of DNA replication sites localized by immunofluorescence and confocal microscopy in MCF-7 cells. *J. Cell. Sci.* **99:** 247–253.

Frappier, L. and M. Zannis-Hadjopoulos. 1987. Autonomous replication of plasmids bearing monkey DNA origin-enriched sequences. *Proc. Natl. Acad. Sci.* **84:** 6668–6672.

Gale, J.M., R.A. Tobey, and J.A. D'Anna. 1992. Localization and DNA sequence of a replication origin in the rhodopsin gene locus of Chinese hamster cells. *J. Mol. Biol.* **224:** 343–358.

Giacca, M., L. Zentilin, P. Norio, S. Diviacco, D. Dimitrova, G. Contreras, G. Biamonti, G. Perini, F. Weighardt, S. Riva, and A. Falaschi. 1994. Fine mapping of a replication origin of human DNA. *Proc. Natl. Acad. Sci.* **91:** 7119–7123.

Gilbert, D.M., H. Miyazawa, and M.L. DePamphilis. 1995. Site-specific initiation of DNA replication in *Xenopus* egg extract requires nuclear structure. *Mol. Cell. Biol.* **15:** 2942–2954.

Gilbert, D.M., H. Miyazawa, F.S. Nallaseth, J.M. Ortega, J.J. Blow, and M.L. DePamphilis. 1993. Site-specific initiation of DNA replication in metazoan chromosomes and the role of nuclear organization. *Cold Spring Harbor Symp. Quant. Biol.* **58:** 475–485.

Guo, Z.-S. and M.L. DePamphilis. 1992. Specific transcription factors stimulate both simian virus 40 and polyomavirus origins of DNA replication. *Mol. Cell. Biol.* **12:** 2514–2524.

Haase, S.B., S.S. Heinzel, and M.P. Calos. 1994. Transcription inhibits the replication of autonomously replicating plasmids in human cells. *Mol. Cell. Biol.* **14:** 2516–2524.

Hand, R. 1978. Eukaryotic DNA: Organization of the genome for replication. *Cell* **15:** 317–325.

Handeli, S., A. Klar, M. Meuth, and H. Cedar. 1989. Mapping replication units in animal cells. *Cell* **57:** 909–920.

Hassan, A.B. and P.R. Cook. 1993. Visualization of replication sites in unfixed human cells. *J. Cell Sci.* **105:** 541–550.

———. 1994. Commentary: Does transcription by RNA polymerase play a direct role in the initiation of replication? *J. Cell Sci.* **107:** 1381–1387.

Hassan, A.B., R.J. Errington, N.S. White, D.A. Jackson, and P.R. Cook. 1994. Replication and transcription sites are colocalized in human cells. *J. Cell Sci.* **107:** 425–434.

Hatton, K.S., V. Dhar, E. H. Brown, M.A. Iqbal, S. Stuart, V.T. Didamo, and C.L. Schildkraut. 1988. Replication program of active and inactive gene families in mammalian cells. *Mol. Cell. Biol.* **8:** 2149–2158.

Heintz, N.H. and J.L. Hamlin. 1982. An amplified chromosomal sequence that includes the gene for dihydrofolate reductase initiates replication within specific restriction fragments. *Proc. Natl. Acad. Sci.* **79:** 4083–4087.

———. 1983. *In vivo* effects of cytosine arabinoside on DNA replication in Chinese hamster ovary cells. II. Cytosine arabinoside affects the rate of synthesis but not the pattern of labeling of an amplified chromosomal sequence at the onset of the S period. *Biochemistry* **22:** 3557–3562.

Heintz, N.H. and B.W. Stillman. 1988. Nuclear DNA synthesis in vitro is mediated via stable replication forks assembled in a temporally specific fashion in vivo. *Mol. Cell. Biol.* **8:** 1923–1931.

Heintz, N.H., L. Dailey, P. Held, and N. Heintz. 1992. Eukaryotic replication origins as promoters of bidirectional DNA synthesis. *Trends Genet.* **8:** 376–381.

Heintz, N.H., J.D. Milbrandt, K S. Greisen, and J.L. Hamlin. 1983. Cloning the initiation region of a mammalian chromosomal replicon. *Nature* **302:** 439–441.

Held, P., E. Soultanakis, L. Dailey, T. Kouzarides, N. Heintz, and N.H. Heintz. 1992. Protein-induced alterations in DNA structure at the dhfr origin of replication. *Colloq. Ges. Biol. Chem. Mosbach* **43:** 113–126.

Ho, L., V.A. Bohr, and P.C. Hanawalt. 1989. Demethylation enhances removal of pyrimidine dimers from the overall genome and from specific DNA sequences in Chinese hamster ovary cells. *Mol. Cell. Biol.* **9:** 1594–1603.

Huberman, J.A. and A.D. Riggs. 1968. On the mechanism of DNA replication in mammalian chromosomes. *J. Mol. Biol.* **32:** 327–341.

Ishimi, Y., K. Matsumoto, and R. Ohba. 1994. DNA replication from initiation zones of mammalian cells in a model system. *Mol. Cell. Biol.* **14:** 6489–6496.

Jacob, F., S. Brenner, and F. Cuzin. 1964. On the regulation of DNA replication in bacteria. *Cold Spring Harbor Symp. Quant. Biol.* **28:** 329–348.

Kill, I.R., J.M. Bridger, K.H.S. Campbell, G. Maldonado-Codina, and C.J. Hutchinson. 1991. The timing of the formation and usage of replicase clusters in S-phase nuclei of human diploid fibroblasts. *J. Cell Sci.* **100:** 869–876.

Kitsberg, D., S. Selig, I. Keshet, and H. Cedar. 1993. Replication structure of the human β-globin domain. *Nature* **366:** 588–590.

Kowalski, D. and M.J. Eddy. 1989. The DNA unwinding element: A novel *cis*-acting component that facilitates opening of the *Escherichia coli* replication origin. *EMBO J.* **8:** 4335–4344.

Krysan, P.J. and M.P. Calos. 1991. Replication initiates at multiple locations on an autonomously replicating plasmid in human cells. *Mol. Cell. Biol.* **11:** 1464–1472.

Krysan, P.J., S.B. Haase, and M.P. Calos. 1989. Isolation of human sequences that replicate autonomously in human cells. *Mol. Cell. Biol.* **9:** 1026–1033.

Leonhardt, H., A.W. Page, H.-U. Weier, and T.H. Bestor. 1992. A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. *Cell* **71:** 865–873.

Leu, T.-H. and J.L. Hamlin. 1989. High-resolution mapping of replication fork movement through the amplified dihydrofolate reductase domain in CHO cells by in-gel renaturation analysis. *Mol. Cell. Biol.* **9:** 523–531.

Levenson, V. and J.L. Hamlin. 1993. A general protocol for evaluating the specific effects of DNA replication inhibitors. *Nucleic Acids Res.* **12:** 3997–4004.

Little, R.D., T.H.K. Platt, and C.L. Schildkraut. 1993. Initiation and termination of DNA replication in human ribosomal RNA genes. *Mol. Cell. Biol.* **13:** 6600–6613.

Looney, J.E. and J.L. Hamlin. 1987. Isolation of the amplified dihydrofolate reductase domain from methotrexate-resistant Chinese hamster ovary cells. *Mol. Cell. Biol.* **7:** 569–577.

Mastrangelo, I.A., P.G. Held, L. Dailey, J.S. Wall, P.V.C. Hough, N. Heintz, and N.H. Heintz. 1993. RIP60 dimers and multiples of dimers assemble link structures at an origin of bidirectional replication in the dihydrofolate reductase amplicon of Chinese hamster ovary cells. *J. Mol. Biol.* **232:** 766–778.

Masukata, H., H. Satoh, C. Obuse, and T. Okazaki. 1993. Autonomous replication of human chromosomal DNA fragments in human cells. *Mol. Biol. Cell* **4:** 1121–1132.

McWhinney, C. and M. Leffak. 1990. Autonomous replication of a DNA fragment containing the chromosomal replication origin of the human c-*myc* gene. *Nucleic Acids Res.* **18:** 1233–1242.

Milbrandt, J.D., J.C. Azizkhan, K.S. Greisen, and J.L. Hamlin. 1983. Organization of a Chinese hamster ovary dihydrofolate reductase gene identified by phenotypic rescue. *Mol. Cell. Biol.* **3:** 1266–1273.

Milbrandt, J.D., N.H. Heintz, W.C. White, S.M. Rothman, and J.L. Hamlin. 1981. Methotrexate-resistant Chinese hamster ovary cells have amplified a 135-kilobase-pair

region that includes the gene for dihydrofolate reductase. *Proc. Natl. Acad. Sci.* **78:** 6043–6047.

Mills, A.D., J.J. Blow, J.G. White, W.B. Amos, D. Wilcock, and R.A. Laskey. 1989. Replication occurs at discrete foci spaced throughout nuclei replicating in vitro. *J. Cell Sci.* **94:** 471–477.

Mitchell, P.J., A.M. Carothers, J.H. Han, J.D. Harding, E. Kas, L. Venolia, and L.A. Chasin. 1986. Multiple transcription start sites, DNase I-hypersensitive sites, and an opposite-strand exon in the 5′ region of the CHO *dhfr* gene. *Mol. Cell. Biol.* **6:** 425–440.

Nakamura, H., T. Morita, and C. Sato. 1986. Structural organization of replicon domains during DNA synthesis phase in the mammalian nucleus. *Exp. Cell Res.* **165:** 291–297.

Nielson T., D. Bell, C. Lamoureux, M. Zannis-Hadjopoulos, and G. Price. 1994. A reproducible method for identification of human genomic DNA autonomously replicating sequences. *Mol. Gen. Genet.* **242:** 280–288.

Orr-Weaver, T.L., C.G. Johnston, and A.C. Spradling. 1989. The role of ACE3 in *Drosophila* chorion gene amplification. *EMBO J.* **8:** 4153–4162.

Pack, R.A. and T. Tsurimoto. 1994. Isolation of human DNA-unwinding elements as sites of DNA polymerase α/primase entry. *Gene* **148:** 277–284.

Pardoll, D.M., B. Vogelstein, and D.S. Coffey. 1980. A fixed site of DNA replication in eukaryotic cells. *Cell* **19:** 527–536.

Pederson, D.S. and N.H. Heintz. 1994. *Transcription factors and DNA replication.* R.G. Landes, Austin, Texas.

Saitoh, Y. and U.K. Laemmli. 1994. Metaphase chromosome structure: Bands drive from a differential folding path of the highly AT-rich scaffold. *Cell* **76:** 609–622.

Stillman, B. 1994. Initiation of chromosomal DNA replication in eukaryotes. Lessons from lambda. *J. Biol. Chem.* **269:** 7047–7050.

Stolzenburg, F., R. Gerwig, E. Dinkl, and F. Grummt. 1994. Structural homologies and functional similarities between mammalian origins of replication and amplification promoting sequences. *Chromosoma* **103:** 209–214.

Taira, T., M. Sanae, M. Iguchi-Ariga, and H. Ariga. 1994. A novel DNA replication origin identified in the human heat shock protein 70 gene promoter. *Mol. Cell. Biol.* **14:** 6386–6397.

Tasheva, E.S. and D.J. Roufa. 1994a. A mammalian origin of bidirectional DNA replication within the Chinese hamster *RPS14* locus. *Mol. Cell. Biol.* **14:** 5628–5635.

———. 1994b. Densely methylated DNA islands in mammalian chromosomal replication origins. *Mol. Cell. Biol.* **14:** 5636–5644.

Vassilev, L.T. and M.L. DePamphilis. 1992. Guide to identification of origins of DNA replication in eukaryotic cell chromosomes. *Crit. Rev. Biochem. Mol. Biol.* **27:** 445–472.

Vassilev, L.T. and E.M. Johnson. 1990. An initiation zone of chromosomal DNA replication located upstream of the *c-myc* gene in proliferating HeLa cells. *Mol. Cell. Biol.* **10:** 4899–4904.

Vassilev, L.T., W.C. Burhans, and M.L. DePamphilis. 1990. Mapping an origin of DNA replication at a single copy locus in exponentially proliferating mammalian cells. *Mol. Cell. Biol.* **10:** 4685–4689.

Vaughn, J.P., P.A. Dijkwel, and J.L. Hamlin. 1990. Replication initiates in a broad zone in the amplified CHO dihydrofolate reductase domain. *Cell* **61:** 1075–1087.

Virta-Pearlman, V.J., P.H. Gunaratne, and A.C. Chinault. 1993. Analysis of a replication

initiation sequence from the adenosine deaminase region of the mouse genome. *Mol. Cell. Biol.* **13:** 5931–5942.

Waga, S. and B. Stillman. 1994. Anatomy of a DNA replication fork revealed by reconstitution of SV40 replication in vitro. *Nature* **369:** 207–212.

Zannis-Hadjopoulos, M., T.O. Nielsen, A. Todd, and G.B. Price. 1995. Autonomous replication in vivo and in vitro of clones spanning the region of the DHFR origin of bidirectional replication (oriβ). *Gene* **151:** 273–277.