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Identification of an Origin of Bidirectional DNA Replication in Mammalian Chromosomes

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Summary

Mechanistically, an origin of bidirectional DNA replication (OBR) can be defined by the transition from discontinuous to continuous DNA synthesis that must occur on each template strand at the site where replication forks originate. This results from synthesis of Okazaki fragments predominantly on the retrograde arms of forks. We have identified these transitions at a specific site within a 0.45 kb sequence approximately 17 kb downstream from the 3′ end of the dihydrofolate reductase gene in Chinese hamster ovary chromosomes. At least 80% of the replication forks in a 27 kb region emanated from this OBR. Thus, initiation of DNA replication in mammalian chromosomes uses the same replication fork mechanism previously described in a variety of prokaryotic and eukaryotic genomes, suggesting that mammalian chromosomes also utilize specific *cis*-acting sequences as origins of DNA replication.

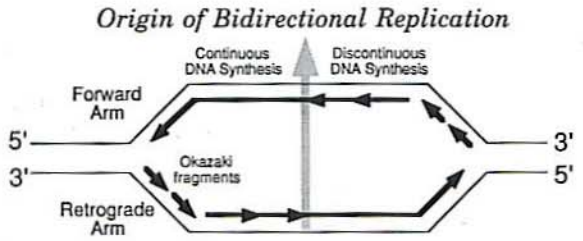
Introduction

Initiation of DNA replication in a number of bacterial, plasmid, bacteriophage, and animal virus genomes is mediated through the interaction of specific proteins with a unique, genetically defined DNA sequence commonly referred to as the origin of replication ("ori"). This interaction serves to unwind the DNA templates and initiate DNA synthesis. In eukaryotic cell chromosomes, replication can then proceed by two possible mechanisms. In the replication fork mechanism, DNA synthesis occurs concomitantly on both DNA template strands as rapidly as they are unwound. Since the two template strands are antiparallel, and all DNA polymerases synthesize DNA only in the 5′ to 3′ direction, the direction of synthesis on one template must be opposite that of fork movement (retrograde). This is accomplished by the repeated initiation of short nascent DNA chains, referred to as "Okazaki fragments," that are eventually joined to the 5′ ends of long nascent DNA chains (Figure 1, Discontinuous DNA Synthesis). When DNA replication proceeds bidirectionally from specific sites, the resulting transition from discontinuous to continuous DNA synthesis that occurs on each strand of DNA defines an origin of bidirectional replication (OBR) as diagrammed in Figure 1.

OBRs have been identified in the mammalian viruses SV40 (Hay and DePamphilis, 1982) and polyomavirus (Hendrickson et al., 1987a, 1987b; DePamphilis et al., 1988), as well as in the prokaryotic genomes of Escherichia coli (Kohara et al., 1985; Seufert and Messer, 1987) and the bacteriophages T7 (Rabkin and Richardson, 1988) and λ (Tsurimoto and Matsubara, 1984). In these examples, the OBR coincides closely with *ori*, but it may also occur some distance from *ori*, as suggested from the location of DNA bubbles in the Drosophila chorion gene locus (Delidakis and Kafatos, 1989; Heck and Spradling, 1990). Analysis of DNA replication in vitro has further shown that DNA synthesis on the forward template of SV40 replication forks is carried out by a DNA polymerase–δ/PCNA complex, a highly processive enzyme that lacks DNA primase, suggesting that synthesis on the forward arm is entirely continuous (for reviews see Blow, 1989; Stillman, 1989). The presence of similar proteins in yeast further suggests that the same is true for all eukaryotic replication forks.

Although the replication fork mechanism is often invoked to explain DNA replication in eukaryotic cell chromosomes, only two of its features have ever been demonstrated: the presence of Okazaki fragments and the presence of replication bubbles (DePamphilis and Wassarman, 1980; Benbow et al., 1985; Umek et al., 1989). In fact, the general lack of evidence for replication forks in mammalian chromosomes has suggested a strand separation mechanism as an alternative model (Micheli et al., 1982; Benbow et al., 1985). In this model, DNA templates are unwound in the absence of DNA synthesis, simultaneously exposing both templates as extensive regions of single-stranded DNA. DNA synthesis can then be initiated at many sites throughout both templates, resulting in a mixture of Okazaki fragments and long nascent DNA chains appearing on both DNA strands. Consequently, no transition from discontinuous to continuous DNA synthesis appears at any specific site. Structures consistent with this mechanism have been observed in DNA from a variety of eukaryotes (Micheli et al., 1982; Benbow et al., 1985; Gaudette and Benbow, 1986).

We have attempted to distinguish between these two mechanisms for DNA replication in mammalian chromosomes by searching for an OBR within a single copy sequence. Previous studies with Chinese hamster ovary cells (CHOC 400) containing 1000 tandemly repeated copies of the dihydrofolate reductase (DHFR) gene region revealed that DNA replication begins within a 4.3 kb sequence centered approximately 16 kb downstream from the DHFR gene (Burhans et al., 1986a; Leu and Hamlin, 1989; Anachkova and Hamlin, 1989). Therefore, we searched for an OBR in this locus, using CHO K1 cells containing the original unamplified copy of the DHFR gene region. Our approach was to measure the fraction



Experimental Protocol

1. Synchronize cells at G1/S border
2. Permeabilize cells
3. Label nascent DNA for 1.5 min with [α-^{32}P]dATP & BrdUTP
4. Fractionate DNA by denaturing gel electrophoresis
5. Electroelute Okazaki fragments and high MW DNA
6. Immunoprecipitate nascent DNA with anti-BrdU antibodies
7. Hybridize nascent DNA to cloned ssDNA templates
8. Quantify autoradiographs by densitometry

Figure 1. Experimental Protocol for Identifying an OBR in Mammalian Cells

of Okazaki fragments synthesized on the retrograde arm of replication forks relative to the forward arm by hybridizing labeled Okazaki fragments to cloned single-stranded DNA templates representing unique segments of this region. A similiar strategy was used previously in this laboratory to identify the OBR in SV40 (Hay and DePamphilis, 1982) and polyomavirus (Hendrickson et al., 1987a, 1987b). The results reveal that Okazaki fragments in mammalian chromosomes, like those in SV40 and polyomavirus chromosomes, originate predominantly, if not exclusively, from the retrograde arm of replication forks. Furthermore, the transitions from discontinuous to continuous DNA synthesis that were previously demonstrated to mark an OBR in these viral genomes also exist in mammalian chromosomes. We identified an OBR within a previously described initiation zone for DNA synthesis near the DHFR gene in CHO cells, by demonstrating that at least 80% of the forks in this region originated within a 0.45 kb sequence approximately 17 kb downstream from the 3′ end of the DHFR gene. Thus, mammalian cells can initiate replication at specific DNA sites using a mechanism that generates the same types of replication forks previously recognized only in bacterial, plasmid, bacteriophage, and animal virus genomes.

Results

Experimental Strategy

Assuming that DNA replication in mammalian chromosomes is initiated bidirectionally at a specific site using the replication fork model, hybridization of Okazaki fragments to cloned strand-specific DNA templates that span the initiation site will reveal the expected transitions between discontinuous and continuous DNA synthesis (Figure 1). In discontinuous DNA synthesis, Okazaki fragments are repeatedly initiated, elongated, and joined to the 5′ ends of long nascent DNA strands. Under our experimental conditions, the ligation step occurs within 1 min of completing synthesis (Tseng and Goulian, 1975), resulting in long nascent DNA strands labeled at both ends. However, the amount of label incorporated at each end depends on the kinetic parameters of each step in DNA synthesis, which are subject to change with experimental conditions. Therefore, the template specificity for fragments of high molecular weight nascent DNA is too complex to predict with confidence. In contrast, the replication fork model predicts that Okazaki fragments anneal predominantly, if not exclusively, to the retrograde arm under all conditions of labeling. For this reason, and because of the difficulties in separating long nascent DNA chains from the large excess of unlabeled DNA that interferes with hybridization to immobilized DNA, we used the template specificity of Okazaki fragment synthesis as a reliable assay for detecting an OBR.

Six DNA segments from the initiation locus of the single copy DHFR gene in CHO K1 cells were cloned into M13 phage. These represented unique strand-specific DNA templates from both arms of replication forks within a 27 kb region. CHO K1 cells were synchronized at their G1/S border under conditions that accumulate early replicating intermediates (Decker et al., 1986). Okazaki fragments were then labeled for 1.5 min, by treating the cells with NP40 to make them permeable to dNTPs and releasing them into S phase in the presence of [α-^{32}P]dATP and BrdUTP under conditions that permit faithful continuation of DNA replication events initiated in vivo (for review see DePamphilis and Wassarman, 1982). For example, the same "early labeled fragment" observed by labeling nascent DNA in intact cells (Heintz and Hamlin, 1982) is also labeled first in NP40 treated cells (Burhans et al., 1986a; this paper) and in isolated nuclei (Heintz and Stillman, 1988).

In a randomly proliferating population of cells, the quantity of Okazaki fragments from the DHFR region that could be isolated from a reasonable number of cells was insufficient to detect a hybridization signal. Therefore, it was necessary to synchronize cells at their G1/S boundary in order to obtain a large population of replication forks within the initiation zone. Furthermore, if origins are not

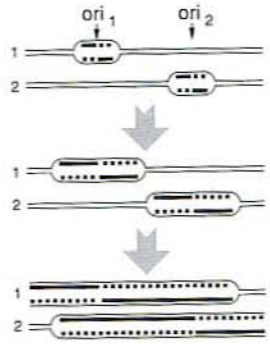


Figure 2. Consequence of Overlapping Replication Bubbles in Mapping Origins of Replication

Discontinuous (broken lines) and continuous (solid lines) DNA synthesis patterns that result when two independent origins of replication (ori) located in separate cells are activated at the onset of S phase. Large arrows indicate passing of time.

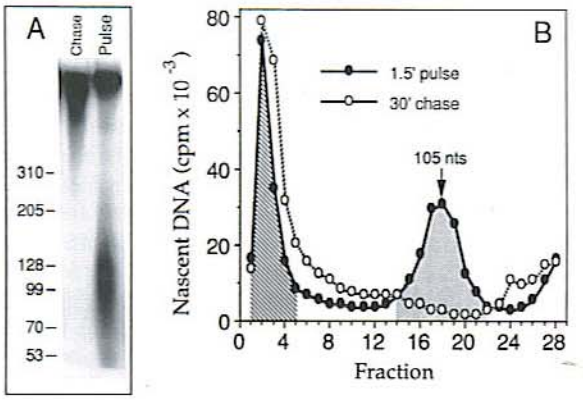


Figure 3. Analysis of Okazaki Fragments Synthesized in NP40-Treated CHO Cells

Synchronized cultures of CHO cells were released into S phase for 3 to 5 min, treated with NP40, and incubated with [α-^{32}P]dATP for 1.5 min (Pulse) or for 1.5 min followed by 30 min in the presence of excess unlabeled dATP (Chase). DNA was purified, heat denatured, and fractionated either in urea–polyacrylamide gels (A), as described by Anderson and DePamphilis (1979), or in alkaline agarose gels (B) (Experimental Procedures). Agarose gels were sliced into equal fractions, and radioactivity was quantified by liquid scintillation counting. Shaded areas correspond to fractions containing high molecular weight DNA (fractions 1–5) and Okazaki fragments (fractions 14–22) that were used in subsequent hybridization experiments.

separated by termination sites in an unsynchronized cell population, a specific sequence may be replicated at any particular time in some of the cells of the population by different origins that lie in adjacent regions, destroying any strand bias that may exist during Okazaki fragment synthesis (Figure 2).

To obtain highly purified populations of Okazaki fragments, DNA was fractionated according to its size by gel electrophoresis, recovered by electroelution, and labeled nascent DNA was separated from unlabeled parental DNA by immunoprecipitating single-stranded Br-DNA with anti-BrdU antibodies. This purification scheme eliminated unlabeled parental DNA strands that could potentially interfere with subsequent hybridization. Nascent DNA was then hybridized to individual complementary template strands that had been cloned into single-stranded M13 DNA and immobilized on nitrocellulose membranes (dot blots). The orientation of template strands was determined either from the directionality of restriction fragments used in cloning or by sequence analysis of the cloned DNA (Caddle et al., 1990). Hybridization signals were quantified by scanning densitometry of autoradiographs. Control experiments demonstrated that the response of the film to radioactive decay was linear within the range of signals obtained.

Identification of Okazaki Fragments

One characteristic of Okazaki fragments in mammalian cells is their size, which ranges from 25 to 300 nucleotides (DePamphilis and Wassarman, 1980). To determine whether pulse-labeled DNA synthesized in CHO K1 cells was of this size, DNA pulse-labeled for 1.5 min was purified, denatured, and then fractionated by electrophoresis in denaturing 5% polyacrylamide–urea gels (Figure 3A). Two size classes of pulse-labeled nascent DNA were observed. One was high molecular size (>5000 bp) and remained at the gel's origin, and the other was a broad peak of low molecular weight DNA. The median weight/average length of these molecules was 105 nucleotides, with a range of 40–250 nucleotides, as expected if these molecules are Okazaki fragments.

A second characteristic of Okazaki fragments is their transient nature; after synthesis, they are rapidly ligated to the long growing chains of nascent DNA (Tseng and Goulian, 1975). When DNA was pulse-labeled for 1.5 min and then incubated for 30 min with an excess of unlabeled dATP ("chase"), at least 90% of the small molecular weight DNA was converted into high molecular weight DNA (Figure 3A), consistent with their designation as Okazaki fragments. The possibility that these DNA fragments resulted from dUTP incorporation and subsequent dUTP glycosylase activity (Grafstrom et al., 1978) is excluded by the fact that the fraction of Okazaki fragments observed by heat denaturation (Figure 3A) was equivalent to the fraction observed by alkaline denaturation (Figure 3B).

A third characteristic of Okazaki fragments is that they originate predominantly, if not exclusively, from the retrograde template (see Figure 1). This results from the antiparallel organization of the two strands in DNA and the fact that all DNA polymerases synthesize nascent DNA

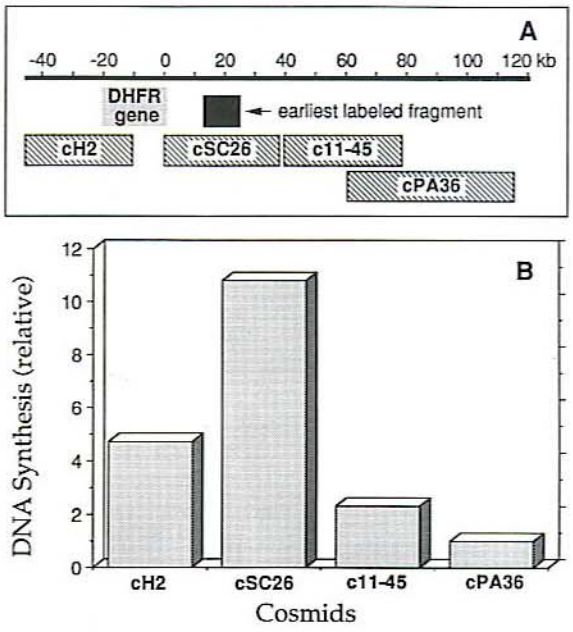


Figure 4. Hybridization of Okazaki Fragments to Large DNA Segments in the DHFR Gene Region

Okazaki fragments were labeled at the onset of S phase as described in Figure 3. Purified Okazaki fragment [^{32}P]DNA was hybridized to cosmid DNA clones containing sequences spanning the genomic region containing the DHFR gene (A) and the relative amounts of hybridization shown in (B). The earliest labeled fragment identified in CHOC 400 cells by Burhans et al. (1986a) is indicated by a solid box.

in one direction (5′ to 3′). Thus, although synthesis of Okazaki fragments on the forward arm is neither required nor excluded, any template preference observed by Okazaki fragments must be for the retrograde arm. As described below, the short, transient nascent DNA chains isolated from CHO K1 cells hybridized predominantly to the predicted retrograde template. This prediction was based on the position of the origin from previous results (see Figure 7) and confirmed by identification of an OBR. Accordingly, they are referred to as Okazaki fragments.

Distribution of Okazaki Fragments in the DHFR Gene Locus

Previous studies have shown that replication of the amplified DHFR gene in CHOC 400 cells containing 1000 copies of the DHFR locus begins at a specific site downstream from the DHFR gene (Heintz and Hamlin, 1982; Burhans et al., 1986a, 1986b; Heintz and Stillman, 1988; Leu and Hamlin, 1989; Anachkova and Hamlin, 1989). To determine whether these observations also hold true in CHO K1 cells containing two copies of this locus, Okazaki fragments were labeled in synchronized cultures 3 to 5 min after release of cells into S phase and then hybridized to cosmid DNA-containing sequences extending over more than 150 kb of the DHFR locus (Figure 4A). The largest hybridization signal was obtained from the cosmid clone (cSC26) containing the earliest labeled fragment identified in CHOC 400 cells (Burhans et al., 1986a, 1986b; Figure 4B). This signal did not result from a nonrandom distribution of repetitive DNA, because repetitive DNA is distributed randomly throughout this region (Foreman and Hamlin, 1989) and DNA from unsynchronized cells hybridized equally well to each cosmid (data not shown; Anachkova and Hamlin, 1989; Ma et al., 1990). These results show that the DHFR initiation locus identified in CHOC 400 cells also functions in CHO K1 cells, and that Okazaki fragments labeled near the onset of S phase were enriched for sequences from this locus, consistent with the presence of an OBR within cSC26.

The distribution of Okazaki fragments between the two arms of replication forks in the DHFR initiation locus was determined by hybridizing them to unique template DNA segments cloned into M13 vectors (Figure 5). Six template segments (A to F) were chosen to span a 27 kb region that contained the "earliest labeled DNA fragment" identified in previous studies (Burhans et al., 1986a, 1986b). Duplicate samples of each M13 clone, as well as duplicate samples of M13 vector DNA that did not contain CHO K1 sequences, were immobilized on membrane supports (dot blots). The amount of DNA in each dot that corresponded to CHO K1 sequences was at least 1000-fold greater than the expected mass of homologous sequences present in the DNA probe. DNA on the dot blots was hybridized with large scale preparations of either ^{32}P-labeled Okazaki fragments or high molecular weight DNA, or with randomly labeled M13 [^{32}P]DNA. Blots were subsequently

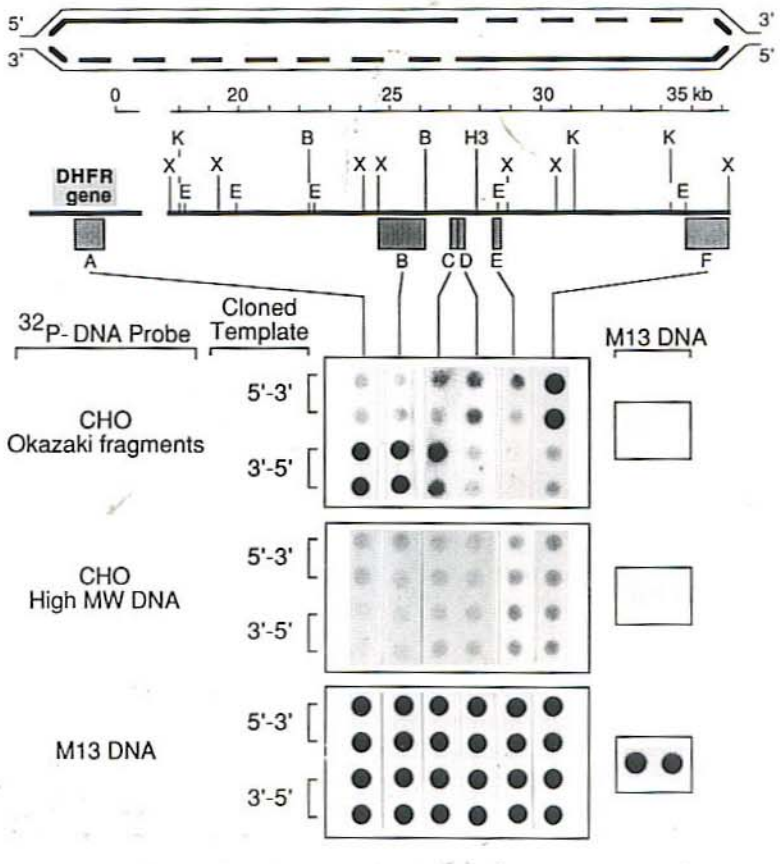


Figure 5. Hybridization of Nascent DNA Chains to Complementary Strands of DNA Segments in the DHFR Gene Region

[^{32}P]DNA probes consisting of Okazaki fragment CHO DNA and high molecular weight CHO DNA were labeled at the onset of S phase as described in Figure 3. Purified [^{32}P]DNA was then hybridized to M13 DNA cloned templates representing either the 5′-3′ or 3′-5′ complementary strands of DNA segments A through F. M13 vector [^{32}P]DNA was used to measure the relative amounts of cloned template available for hybridization. These three [^{32}P]DNA probes were also hybridized with M13 vector DNA. Each of the cloned templates as well as the M13 vector DNA were applied to the membrane in duplicate.

The proximity to the DHFR gene of segments A through F is indicated in reference to restriction sites XbaI (X), KpnI (K), EcoRI (E), BamHI (B), and Hind3 (H). Segment A is a 1 kb sequence corresponding to DHFR cDNA. Segments B (1.6 kb), C (230 bp), D (250 bp), and E (355 bp) were subcloned from S13X-24 (Burhans et al., 1986a) into M13mp18 and mp19 using standard techniques (Sambrook et al., 1989). Segments C and D correspond to Hae3 fragments from nucleotides 2431 to 2661 and 2662 to 2914, respectively, in the 6157 bp region sequenced by Caddle et al. (1990), and segment E corresponds to nucleotides 3947 to 4301. Segment F (1.2 kb) corresponds to an EcoRI–XbaI fragment subcloned from cosmid cSC26 (Looney and Hamlin, 1987). Orientation was determined by sequence analysis and by the polarity of restriction sites used in cloning.

washed under high stringency conditions, exposed to X-ray film, and the hybridization signals obtained were quantified by densitometry.

In each of the six clones, ^{32}P-labeled Okazaki fragments hybridized preferentially to the predicted retrograde templates (Figure 5). In contrast, signals obtained with CHO K1 high molecular weight [^{32}P]DNA probe were either equivalent for complementary strand templates or slightly biased to the predicted forward DNA template (Figure 5), as previously observed with SV40 and polyomavirus replicating DNA (Perlman and Huberman, 1977; Kaufmann et al., 1978; Cusick et al., 1981; Hendrickson et al., 1987b). Similarly, ^{32}P-labeled DHFR region DNA isolated from recombinant plasmids containing segments A and F annealed equally well to both template strands (data not shown). In addition, M13 [^{32}P]DNA probe annealed equally well to each of the cloned template DNAs, confirming that equivalent amounts of the template sequences immobilized on the membrane were available for hybridization. Therefore, the biases observed with ^{32}P-labeled Okazaki fragments were specific for the Okazaki fragment fraction of the pulse-labeled DNA.

Two observations indicate that the hybridization signals observed with CHO K1 DNA probe represented sequence-specific hybridization. First, little or no hybridization was observed to M13 DNA. Second, the total signal from both strands in each matched set were generally proportional to the size of the cloned segment. These results reveal that Okazaki fragments are synthesized predominantly on one side of replication forks in CHO K1 cells.

Identification of an OBR

The hybridization biases observed with clones A through C were to the 3′-5′ template, whereas the biases observed with clones D through F were to the 5′-3′ template (Figure 5). This transition between the presence and absence of Okazaki fragments on each template strand defines an OBR within segments C and D. Data from several independent experiments were quantified and averaged together (Figure 6). Since the strength of the sequence-specific signal observed was proportional to the length of the cloned CHO K1 DNA segment (data not shown), the fraction of the signal due to nonspecific hybridization with M13 vector DNA increased with decreasing probe length. This background was significant only with the two smallest probes (Figure 6, probes C and D). These data revealed that Okazaki fragments annealed, on average, three (not corrected for background) or four (corrected for background) times more frequently to the retrograde template than to the forward template, consistent with similar studies on SV40 and polyomavirus replication forks (Hendrickson et al., 1987b). Therefore, 80% of replication forks originated from a 0.45 kb locus that maps approximately 17 kb downstream from the 3′ end of the DHFR gene, identifying an OBR within this locus. Hybridization of Okazaki fragments labeled in synchronized or unsynchronized CHOC 400 cells containing 1000 copies of the DHFR locus yielded results similar to those described above for single copy CHO K1 cells.

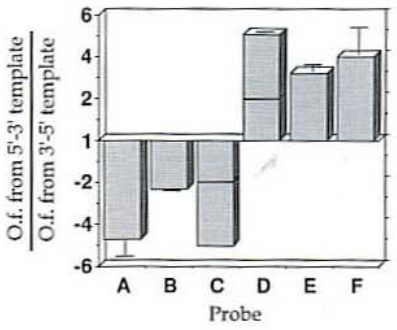


Figure 6. Identification of an OBR in the DHFR Locus of CHO Cells

The hybridization signals for Okazaki fragments synthesized in CHO and CHOC 400 cells were quantified by densitometry. The ratios between complementary strands of DNA segments A through F were calculated for two to nine independent experiments, and the average values ± standard error of the mean were plotted. Autoradiographs were selected in which the intensity of the signal was proportional to the amount of [^{32}P]DNA present. This was determined using known amounts of [^{32}P]DNA spotted on a membrane. For radioactivity between 20 and 100 cherenkov cpm per dot, exposure times of ~20 hr revealed a linear response. The line across the bar in probes C and D represents the ratio calculated before substraction of nonspecific hybridization to M13 vector DNA; background for the other probes was always undetectable.

Inability of Origin Sequences to Promote Replication of Plasmid DNA

In a variety of prokaryotic and eukaryotic genomes, OBRs include unique *cis*-acting sequences that are necessary for initiation of DNA replication. In general, these sequences promote autonomous replication of plasmid DNA when plasmids are provided with the necessary *trans*-acting factors either in vivo or in vitro. Therefore, three DNA fragments containing the DHFR region OBR were tested for their ability to promote autonomous replication in circular DNA molecules: cosmid cSC26 (see Figure 4), a plasmid containing a 5.4 kb fragment spanning the BamHI site 16 kb from the DHFR gene to the next KpnI site, and a plasmid containing a 4.3 kb XbaI fragment from the XbaI site at 14.5 kb to the next XbaI site. Sequences that did not contain the origin region defined in this study were also evaluated. These included pML-1 vector DNA, cosmid cH2 DNA (see Figure 4), a plasmid containing a 4.8 kb fragment extending from the XbaI site 9 kb from the DHFR gene to the next XbaI site (see Figure 5), ors12 (Frappier and Zannis-Hadjopoulos, 1987), and pARS65 (Iguchi-Ariga et al., 1987).

Replication activity was first tested by transfecting from 10 ng to 10 μg of each DNA into ~10^6 CHO K1, HeLa, 293, or HL-60 cells. HeLa and 293 cells are transformed human cell lines that replicate SV40 DNA efficiently in vivo and in vitro. Human HL-60 cells contain high levels of c-myc protein and have been reported to replicate putative mouse and human origin sequences. Transfection was performed using calcium phosphate precipitation (Wigler et al., 1978), DEAE dextran (Guo et al., 1989), and liposome fusion (Felgner et al., 1987) protocols. Replication was measured between 24 and 96 hr posttransfection using resistance of newly replicated DNA to cleavage by DpnI as an assay for a single round of DNA replication

(Peden et al., 1980). DNA replication was also evaluated by incubating circular DNA molecules in extracts of each cell line as described by Decker et al., 1987; Li and Kelly, 1985; or Iguchi-Ariga et al., 1987.

In general, none of the cosmid or plasmid DNAs was observed to replicate under any of these conditions. Our level of detection was 1 pg of DNA per 10^6 cells. In contrast, plasmids containing the SV40 origin and T-ag gene always replicated efficiently when either transfected concurrently into HeLa cells or incubated in parallel extracts of HeLa, 293, or HL-60 cells. Occasionally, however, small amounts of DpnI-resistant material (~10 pg/10^6 cells) were observed with both ori$^+$ and ori$^-$ DNA molecules, particularly in calcium phosphate transfections or when cosmid DNA was replicated in extracts of 293 cells. Regardless of the mechanism by which this replication occurred, it did not require the OBR identified in our experiments.

Discussion

DNA Replication in Mammalian Chromosomes Begins within a Limited Initiation Locus

Initial efforts to locate the site where DNA replication begins in the DHFR gene region used CHOC 400 cells, containing 1000 amplified copies of the DHFR domain synchronized at the G1/S border by isoleucine starvation and arrest of DNA synthesis with metabolic inhibitors (Figure 7). Heintz and Hamlin (1982) identified three specific DNA fragments that rapidly incorporated radioactive precursors when cells were released into S phase. One of these early labeled fragments is located between 12 and 24 kb downstream of the DHFR gene and was later shown to replicate first (Burhans et al., 1986a; Heintz and Stillman, 1988). Hybridization of labeled DNA to unique genomic sequences localized the earliest replicating DNA to a 4.3 kb sequence (Burhans et al., 1986a, 1986b). Further technical improvements ("in-gel renaturation") resolved the position of this initiation locus to about 1.8 kb (Leu and Hamlin, 1989), later refined to approximately 500 bp (Anachkova and Hamlin, 1989). Additional initiation loci may also exist further downstream of the DHFR gene (Leu and Hamlin, 1989; Anachkova and Hamlin, 1989; Handeli et al., 1989).

A new approach for mapping OBRs at single copy loci was reported recently based on the assumption that prefork histones segregate to the forward arm of replication forks (Handeli et al., 1989). In these experiments, exponentially proliferating CHO K1 cells were incubated for 24 hr in the presence of a protein synthesis inhibitor, and then nuclei were digested with micrococcal nuclease to destroy all unprotected nascent DNA. The results were consistent with an OBR contained within a 15 kb segment that encompasses the initiation zone described in CHOC 400 cells (Figure 7). Furthermore, this DNA segment could be moved to other regions of the genome and still act as an ori, consistent with the presence of an *ori* sequence. We have recently confirmed these observations, but in our experiments, presumptive nucleosomes protected the retrograde arm rather than the forward arm:

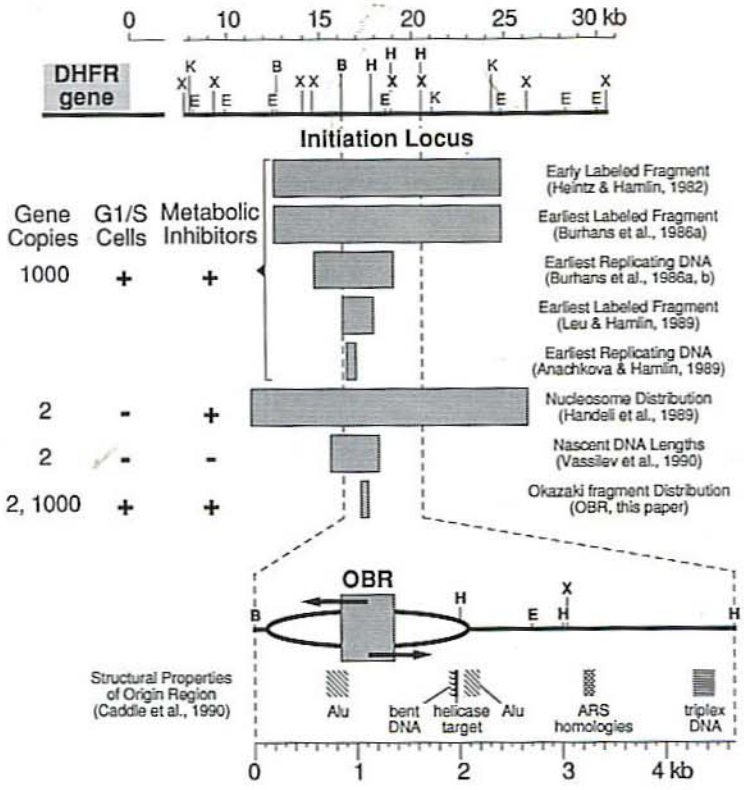


Figure 7. Initiation of DNA Replication in the DHFR Gene Region of CHO and CHOC 400 Cells

The outer limits of the OBR determined in this paper are compared with those of initiation loci (stippled bars) identified by a variety of other methods under various experimental conditions. The proximity of the initiation loci to the DHFR gene is indicated in reference to restriction sites XbaI (X), KpnI (K), EcoRI (E), BamHI (B), and Hind3 (H). Some experiments used CHOC 400 cells containing 1000 copies of the DHFR amplicon, while others used CHO cells containing only the two original copies of this locus (Gene Copies). Some cells were synchronized (+) at the G1/S border using isoleucine deprivation followed by incubation in complete medium containing aphidicolin or fluorodeoxyuridine (Metabolic Inhibitors), while other experiments used nonsynchronized cells (−), treated with the protein synthesis inhibitor emetine (Handeli et al., 1989). The putative helicase DNA binding site indicated along with results of Caddle et al. (1990) is the unpublished data of L. Dailey, M. S. C., N. H., and N. H. H.

Okazaki fragments as well as nascent DNA protected from micrococcal nuclease digestion annealed to the same DNA template (unpublished data). This surprising observation questions the premise that asymmetrical nucleosome segregation is responsible for the observed bias.

In marked contrast to these results, analysis of DNA structures in this region by 2-D gel electrophoresis led to the conclusion that initiation of replication occurs randomly throughout a 30 kb region that includes our OBR (Vaughn et al., 1990). This would mean that, at any point within this 30 kb region, an equal number of forks are traveling in either direction. Under these conditions, one could not distinguish any asymmetry in replication forks from this region of the genome, because forks traveling in one direction cannot be separated from those traveling in the opposite direction. Therefore, the conclusion of Vaughn et al. (1990) is difficult to reconcile with the results of Handeli et al. (1989) and our observations on Okazaki fragments: the majority (80% or greater) of replication forks originate at a specific locus. In addition, their conclusion is contrary to data previously published by several laboratories including those of Vaughn et al. (Figure 7).

How might this paradox be resolved? As Vaughn et al. (1990) suggest, difficulties in quantifying the relative signals of the bubble arcs and fork arcs in different fragments from this locus may have prevented detection of a nonrandom pattern of initiation events. In addition, DNA structures referred to as replication intermediates may not have been correctly identified. The putative replication bubbles in Vaughn et al. (1990) consist of faint signals against a background rich in replication forks, indicating a paucity of initiation events in this 30 kb region relative to the amount of DNA replication activity. Fractionation of cellular DNA by 2-D gel electrophoresis does not identify newly synthesized DNA; it examines total DNA and identifies DNA structures based on theoretical considerations supported by analysis of model systems. While this approach has proven a powerful technique with yeast chromosomes and plasmids, the increased complexity of mammalian chromosomes (10^5 > plasmid, 2×10^2 > yeast) may yield DNA structures not currently understood and that are not transient intermediates in replication. Perhaps the interpretation derived from analysis of simpler genomes should not be superimposed on data from complex genomes until the putative replication structures have been confirmed by other techniques.

In this light, it is worth noting that application of the 2-D gel technique to the amplified Drosophila chorion gene locus, which is ~1000-fold less complex than single copy mammalian sequences, gave results consistent both with those of Vaughn et al. (1990) and those described in this paper. Heck and Spradling (1990) concluded that, although multiple initiation sites may exist within a 12 kb locus, "a model in which usually a single origin fires per strand, and in which the β-origin is preferred 70% to 80% of the time can explain most of the observed replicative intermediates." Thus, one would predict that analysis of the distribution of Drosophila Okazaki fragments would identify an OBR at the β-origin.

Identification of an OBR within the Initiation Locus

In this study, we searched for an OBR in the same DHFR gene region that previous studies suggested contained an ori (Figure 7), by measuring the distribution of Okazaki fragments between the two arms of replication forks (Figure 1). This was accomplished using NP40-treated CHO K1 and CHOC 400 cells to label Okazaki fragments to a high specific activity and then annealing them to cloned DNA templates under highly efficient hybridization conditions. Our data showed that DNA replication through single as well as amplified copies of the DHFR region of a mammalian chromosome was indistinguishable from replication in SV40 and polyomavirus chromosomes, in that Okazaki fragments originated predominantly, if not exclusively, from the retrograde arm of replication forks. Furthermore, the transitions from discontinuous to continuous DNA synthesis that were previously demonstrated to mark an OBR in these viral genomes also marked an OBR within a previously described initiation zone for DNA synthesis near the DHFR gene in CHO cells. About 80% of the replication forks in this region originated within a 0.45 kb sequence approximately 17 kb downstream from the 3′ end of the DHFR gene. Thus, this segment contains an OBR that is active in both the normal and amplified copies of this region. The position of this OBR is consistent with all previously published results identifying an initiation locus in this region of the CHO genome (summarized in Figure 7). Since this initiation locus has been identified in both single copy and multicopy cell lines, in synchronized as well as unsynchronized cells, and in cells treated or untreated with various metabolic inhibitors, it is not likely an artifact of experimental conditions. In particular, Vassilev et al. (1990) have recently shown that CHO K1 cells proliferating under normal physiological conditions initiate DNA replication in a 2 kb segment that contains the OBR described here.

Okazaki fragments may, in fact, originate exclusively from the retrograde arm. About 10% of the pulse-labeled DNA in our experiments migrated as Okazaki fragments even after a 30 min "chase" period (Figure 3B), indicating the presence of fragmented [^{32}P]DNA that would anneal randomly to both template strands. This artifact would reduce the extent of asymmetry measured in these experiments. Furthermore, the appearance of Okazaki fragments from what should be the forward arm of replication forks can result when the same DNA template appears as the retrograde arm of some forks but the forward arm of others. For example, while most SV40 and polyomavirus chromosomes initiate bidirectional replication at the same site, a small fraction replicates unidirectionally. In addition, some molecules that replicate bidirectionally contain deletions between the origin and the normal termination site for replication, resulting in Okazaki fragments being synthesized on both DNA templates through a unique sequence in a population of molecules (discussed in Hendrickson et al., 1987b). In mammalian chromosomes, this would be equivalent to replication forks from one origin passing through an adjacent origin (Figure 2) and genetic alterations that change the distance between adjacent ori-

gins, which may, in turn, increase the frequency of replication through adjacent origins. Small numbers of Okazaki fragments synthesized on forward arms of replication forks could also be explained by a small fraction of initiation events occurring outside the primary OBR, as previously observed during SV40 DNA replication (Martin and Setlow, 1980; Tack and Proctor, 1987) and Drosophila chorion gene amplification (Heck and Spradling, 1990). This could account for the trace amounts of replication bubbles relative to a large amount of replication forks observed throughout a 30 kb region that includes the OBR, as suggested by Vaughn et al. (1990).

One implication of the fact that at least 80% of Okazaki fragments originate from the retrograde arm of forks in exponentially proliferating CHOC 400 cells is that most copies of the OBR in these cells are active during each cell cycle. Otherwise, Okazaki fragments would appear to originate equally from both DNA templates, because replication forks emanating from adjacent origins would travel through an inactive OBR (Figure 2). However, since every amplified initiation locus in CHOC 400 cells is not activated at the same time (Leu and Hamlin, 1989; W. C. B., unpublished data), one can also infer from our results that mammalian cells have some mechanism to prevent forks from entering adjacent replicons. Otherwise, the rate of fork travel would have allowed forks from one origin to replicate through adjacent origins that had not yet been activated.

Initiation of Replication in Mammalian Chromosomes Shares Structural Features with Initiation in Other Genomes

Three different mechanisms have been identified by which duplex DNA is replicated: replication forks, strand separation, and strand displacement. The strand displacement mechanism, exemplified by adenovirus and mitochondrial DNA replication, does not involve synthesis of Okazaki fragments (Stillman, 1989) and, therefore, is not applicable to eukaryotic chromosome replication. The strand separation mechanism, described in the Introduction and suggested as a general model for replicating eukaryotic chromosomes (Micheli et al., 1982; Benbow et al., 1985), is not consistent with the data presented here for Okazaki fragment synthesis in CHO K1 and CHOC 400 cells. These data demonstrate that DNA replication in mammalian chromosomes occurs by the replication fork mechanism described in Figure 1.

Previous studies have suggested that replication forks in mammalian chromosomes are similar, if not identical, to those in SV40 and polyomavirus chromosomes that rely on their cellular host for all proteins involved in replication except viral T-antigen (DePamphilis and Wassarman, 1982; DePamphilis, 1987). Replication in all three genomes involves repeated synthesis of RNA-primed nascent DNA chains with indistinguishable structures that are synthesized by the same enzyme, DNA primase–DNA polymerase-α. In addition, Okazaki fragments synthesized at replication forks in SV40 and polyomavirus originate predominantly, if not exclusively, from the retrograde template. From 75% to 92% of polyomavirus and SV40 Okazaki fragments hybridized specifically to the retrograde template (Hunter et al., 1977; Perlman and Huberman, 1977; Kaufmann et al., 1978; Cusick et al., 1981; Hendrickson et al., 1987b), and 95% to 97% of the RNA primers were located on this template (Hendrickson et al., 1987b), in excellent agreement with the results presented here for mammalian chromosomes.

Does initiation of DNA replication in mammalian chromosomes require *ori* sequences? So far, "autonomously replicating sequences" in yeast (Newlon, 1988) and "amplification control elements" in Drosophila (Delidakis and Kafatos, 1989; Heck and Spradling, 1990) are the only clear examples of *ori* sequences in the replication of eukaryotic chromosomes. However, their mechanism of action is not clear, and the presence or absence of an OBR has not yet been addressed (Kipling and Kearsey, 1990). Replication also originates at specific sites in other eukaryotes (Benbow et al., 1985), and several mammalian DNA sequences have been reported to promote replication of plasmids in mammalian cells (Frappier and Zannis-Hadjopoulos, 1987; Iguchi-Ariga et al., 1987, 1988a, 1988b; Krysan et al., 1989; McWhinney and Leffak, 1990). However, interpretation of these latter results in terms of *ori* sequences is controversial (Laskey et al., 1989; Umek et al., 1989).

In the present study we have shown that at least one mammalian chromosomal initiation locus contains an OBR analogous to those previously identified in SV40 (Hay and DePamphilis, 1982), polyomavirus (Hendrickson et al., 1987a, 1987b), E. coli (Kohara et al., 1985; Seufert and Messer, 1987), bacteriophage T7 (Rabkin and Richardson, 1988), and bacteriophage λ (Tsurimoto and Matsubara, 1984), suggesting that initiation of mammalian chromosomal DNA replication also utilizes *ori* sequences. The fact that the DHFR initiation locus described here also acts as an ori when transferred to other chromosomal locations (Handeli et al., 1989; W. C. B., unpublished data) strongly supports this hypothesis. However, in our hands, this putative *ori*, like others we tested, was not active when placed in circular 3 to 30 kb DNA molecules, suggesting that mammalian origins, in contrast to yeast origins (autonomously replicating sequence elements), may function only in the context of a large chromosome.

Experimental Procedures

Culture and Synchronization of Cells

CHO K1 (American Type Culture Collection) and CHOC 400 (Heintz and Hamlin, 1982) cells were seeded in 150 mm tissue culture dishes and grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and nonessential amino acids. When cell monolayers reached 80%–90% confluency, approximately 90%–95% of the cells were synchronized at the G1/S phase boundary by first collecting them in the G0 phase by isoleucine deprivation as described by Heintz and Hamlin (1982) (except that deprivation was for 36 hr), followed by release into complete medium containing 10 μg/ml aphidicolin for 12 hr. The efficacy of this synchronization regimen was determined by fluorescent-activated cell sorting of propidium iodide–stained nuclei (Vindelov et al., 1983).

Labeling of Nascent Cell DNA

Cells collected at the G1/S boundary were washed three times with ice-cold medium without serum to remove the aphidicolin. Cells were then allowed to transit into S phase by adding warm complete medium and

reincubating them at 37°C for 3 to 5 min. They were washed once again with ice-cold medium without serum. Excess medium was aspirated, and the cells were scraped into the residual medium (~1 ml/150 mm dish per ~2 × 10^7 cells). The cell suspension from each dish (maintained at 0°C to 4°C until the labeling period began) was transferred into a 1.5 ml microcentrifuge tube, and the cells were pelleted by centrifugation for 3 min in a Beckman microfuge (setting 3). Supernatants were aspirated, leaving 50–100 μl of packed cells per tube.

Each 100 μl of packed cells was resuspended in 120 μl of ice-cold 2× replication cocktail (60 mM potassium HEPES [pH 7.8], 0.2 mM each dGTP, dCTP, and BrdUTP, 0.4 mM each GTP, CTP, and UTP, 8 mM ATP, 20 mM $MgCl_2$, 0.2 mg/ml nuclease-free bovine serum albumin, 2 mM dithiothreitol, and 30% [v/v] glycerol). The volume of each suspension was adjusted to 200 μl per tube in order that the temperature of each reaction was equilibrated rapidly at the same rate, and 20 μl of [α-^{32}P]dATP (10 mCi/ml, 3000 Ci/mmol, Amersham) was added to each tube. NP40 was then added to a final concentration of 0.4%. Labeling reactions were started by transferring the tubes to a 34°C water bath. Reactions were stopped after precisely 1.5 min of incubation by adding 800 μl of 50 mM Tris–HCl (pH 7.8), 10 mM EDTA, 0.4 M NaCl, 0.6% sodium dodecyl sulfate, 0.2 mg/ml proteinase K (Boehringer Mannheim). Proteins were digested by incubating reactions for 1 to 2 hr in a 37°C water bath. For chase experiments, dATP was added at the end of the labeling period to give a final concentration of 0.1 mM, and the incubation continued for 30 min, at which point reactions were stopped and proteins were digested as described above.

Isolation and Analysis of DNA

Residual peptides were precipitated by adding 330 μl of saturated NaCl to each 1 ml of lysate. Tubes were gently mixed until an even precipitate formed. Precipitates were removed by centrifugation at 2,000 × g for 15 min at room temperature, and supernatants were combined. DNA was precipitated from the supernatant with 2 vol of absolute ethanol at room temperature. The visible precipitate was collected by centrifuging for 30 min at 10,000 rpm in a Sorvall HB4 rotor at 4°C. The DNA pellet was rinsed twice with 5 to 10 ml of 70% ethanol, redissolved in 10 mM Tris–HCl (pH 7.8) and 1 mM EDTA using 50 μl per 150 mm dish of cells, and then denatured in boiling water for 3 min. DNA was fractionated by electrophoresis through 1.8% agarose alkaline gels (Maniatis et al., 1982) at 2 V/cm for 16 hr at room temperature. A 123 bp ladder of DNA standards (Gibco Life Sciences) was run in a parallel lane and visualized by excising the lane and staining it for 30 min in 300 ml of 30 μg/ml ethidium bromide in 0.5 M Tris–HCl (pH 7.8).

[^{32}P]DNA was localized by covering the wet gels with Saran Wrap and exposing them to individually wrapped Kodak X-Omat AR films for 1 hr through the film wrapping. Appropriate regions of the gel were excised, and the labeled DNA was electroeluted into 0.5 ml of 0.5× TBE buffer (Maniatis et al., 1982) using an electroelution trap (Schleider and Schuell) according to the manufacturer's instructions. Greater than 99% of the DNA was recovered at this step. Contaminating RNA was then hydrolyzed by adjusting the solutions to 0.2 N NaOH and incubating at 37°C for 24 hr. Solutions were neutralized by adding Tris–HCl (pH 7.8) to a final concentration of 100 mM and adjusting the pH to 7.2 with dilute HCl.

Immunoprecipitation of Nascent DNA

The high molecular weight DNA fraction was sonicated to an average length of 300 bp, and both fractions of labeled DNA were heat denatured in boiling water for 5 min and then rapidly cooled to 0°C in an ice bath. Solutions were adjusted to .05% Triton X-100 and .15 M NaCl, and incubated with 10 μl of mouse anti-BrdU monoclonal antibody (Becton-Dickinson, 25 μg/ml) per 150 mm dish of cells for 45 min at 4°C with constant agitation (Vassilev and Russev, 1988). Rabbit IgG directed against mouse IgG (2 μl per dish of 2.3 mg/ml, Sigma) was then added to precipitate BrdU–DNA–Ab complexes under the same conditions. Immunoprecipitates were collected by centrifugation at 4°C for 5 min at 12,000 rpm in an Eppendorf microfuge and then resuspended in 0.2 ml of 10 mM Tris–HCl (pH 7.8) and 1 mM EDTA.

Generally, about 50% of the [^{32}P]DNA was precipitated, and its specific activity was increased by about 100-fold. Typical preparations of labeled, nascent DNA for hybridization analysis were performed with cells from ten 150 mm dishes. Yields of labeled Okazaki fragments or high molecular weight DNA were at least 1 × 10^6 cherenkov cpm per ten 150 mm dishes of cells or ~2 × 10^8 cells.

Blotting and Hybridization of DNA

M13 clones of various DNA segments from the DHFR initiation locus (Figure 5) were subcloned from previously constructed plasmids (Burhans et al., 1986a) or from cosmid DNA (Looney and Hamlin, 1987). M13 virus stocks were propagated and large scale preparations of cloned ssDNA prepared from virions as described previously (Hay and DePamphilis, 1982), except that deproteinized virion DNA was fractionated by electrophoresis in 0.8 agarose/TBE gels run at 2 V/cm for 16 hr, and the full-length circular molecules were electroeluted into 0.5× TBE. They were then precipitated with ethanol and redissolved in 10 mM Tris–HCl (pH 7.8) and 1 mM EDTA. Complementary strand clones were "C-tested" as described in Howarth et al. (1981) to confirm their complementary nature.

Blots were prepared using M13 cloned DNA or sonicated cosmid DNA dissolved in 0.4 N NaOH at 6 μg/ml. Each solution (0.5 ml) was applied in duplicate to prewetted Zeta-Probe membranes (Bio–Rad) using a Schleicher and Scheull dot blot manifold. The membranes were rinsed briefly in 2× SSC (Maniatis et al., 1982), air dried, and baked under vacuum for 0.5 hr at 80°C.

Membranes containing immobilized cosmid or M13 DNA were incubated in 5 ml of prehybridization buffer (Church and Gilbert, 1984). The large excess of inorganic phosphate in this buffer improves hybridization efficiency (Mahmoudi and Lin, 1989). Labeled Okazaki fragments and high molecular weight DNA (0.2 ml samples) were denatured in boiling water for 3 min and then added directly to the prehybridization buffer. For M13 [^{32}P]DNA probes, M13 vector DNA was labeled with [α-^{32}P]dCTP by random primer extension (Feinberg and Vogelstein, 1984), heat denatured, and then added to the prehybridization buffer (final concentration = 10^6 cpm/ml). Hybridization was carried out for 16 hr at 65°C with agitation. Membranes were then washed in 2× SSC and 2% SDS once at room temperature for 5 min and once at 68°C for 0.5 hr. This was followed by washing them in 0.2× SSC and 0.2% SDS once at 68°C for 0.5 hr and five times at room temperature for 1 min. [^{32}P]DNA was detected by exposing membranes to Kodak X-Omat AR film with a Dupont Cronex Lightning Plus intensifying screen for up to 2 weeks at −80°C, and autoradiographic signals were quantified by densitometry.

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References

Anachkova, B., and Hamlin, J. L. (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.

Anderson, S., and DePamphilis, M. L. (1979). Metabolism of Okazaki fragments during simian virus 40 DNA replication. J. Biol. Chem. *254*, 11495–11504.

Benbow, R. M., Gaudette, M. F., Hines, P. J., and Shioda, M. (1985). Initiation of DNA replication in eukaryotes. In Control of Animal Cell Proliferation, Vol. I, A. L. Boynton and H. L. Leffert, eds. (New York: Academic Press), pp. 449–483.

Blow, J. (1989). Eukaryotic chromosome replication requires both α and δ DNA polymerases. Trends Genet. *5*, 134–136.

Burhans, W. C., Selegue, J. E., and Heintz, N. H. (1986a). Isolation of the origin of replication associated with the amplified Chinese hamster dihydrofolate reductase domain. Proc. Natl. Acad. Sci. USA *83*, 7790–7794.

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

Caddle, M. S., Lussier, R. H., and Heintz, N. H. (1990). 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.

Church, G. M., and Gilbert, W. (1984). Genomic sequencing. Proc. Natl. Acad. Sci. USA *81*, 1991–1995.

Cusick, M. E., Herman, T. M., DePamphilis, M. L., and Wassarman, P. M. (1981). Structure of chromatin at DNA replication forks: prenucleosomal DNA is rapidly excised from replicating simian virus 40 chromosomes by micrococcal nuclease. Biochemistry *20*, 6648–6658.

Decker, R. S., Yamaguchi, M., Possenti, R., and DePamphilis, M. L. (1986). Initiation of SV40 DNA replication *in vitro*: aphidicolin causes accumulation of early replicating intermediates and allows determination of the initial direction of DNA synthesis. Mol. Cell. Biol. *6*, 3815–3825.

Decker, R. S., Yamaguchi, M., Possenti, R., Bradley, M., and DePamphilis, M. L. (1987). In vitro initiation of DNA replication in SV40 chromosomes. J. Biol. Chem. *262*, 10863–10872.

Delidakis, C., and Kafatos, F. C. (1989). Amplification enhancers and replication origins in the autosomal chorion gene cluster of Drosophila. EMBO J. *8*, 891–901.

DePamphilis, M. L. (1987). Replication of simian virus 40 and polyoma virus chromosomes. In Molecular Aspects of Papovaviruses, Y. Aloni, ed. (Boston: Martinus Nijhoff), pp. 1–40.

DePamphilis, M. L., and Wassarman, P. M. (1980). Replication of eukaryotic chromosomes: a close-up of the replication fork. Annu. Rev. Biochem. *49*, 627–666.

DePamphilis, M. L., and Wassarman, P. M. (1982). Organization and replication of papovavirus DNA. In Organization and Replication of Viral DNA, A. S. Kaplan, ed. (Boca Raton, Florida: CRC Press), pp. 37–114.

DePamphilis, M. L., Martinez-Salas, E., Cupo, D. Y., Hendrickson, E. A., Fritze, C. E., Folk, W. R., and Heine, U. (1988). Initiation of polyomavirus and SV40 DNA replication, and the requirements for DNA replication during mammalian development. Cancer Cells *6*, 165–175.

Feinberg, A. P., and Vogelstein, B. (1984). Addendum: a technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. *137*, 266.

Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M., and Danielsen, M. (1987). Lipofection: a highly efficient lipid mediated DNA transfection procedure. Proc. Natl. Acad. Sci. USA *84*, 7413–7417.

Foreman, P. K., and Hamlin, J. L. (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.

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

Gaudette, M. F., and Benbow, R. M. (1986). Replication forks are underrepresented in chromosomal DNA of *Xenopus laevis* embryos. Proc. Natl. Acad. Sci. USA *83*, 5953–5957.

Grafstrom, R. H., Tseng, B. Y., and Goulian, M. (1978). The incorporation of uracil into animal cell DNA in vitro. Cell *15*, 131–140.

Guo, Z.-S., Gutierrez, C., Heine, U., Sogo, J. M., and DePamphilis, M. L. (1989). Origin auxiliary sequences can facilitate initiation of simian virus 40 DNA replication in vitro as they do in vivo. Mol. Cell. Biol. *9*, 3593–3602.

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

Hay, R. T., and DePamphilis, M. L. (1982). Initiation of SV40 DNA replication in vivo: location and structure of 5′ ends of DNA synthesized in the *ori* region. Cell *28*, 767–779.

Heck, M. M. S., and Spradling, A. C. (1990). Multiple replication origins are used during Drosophila chorion gene amplification. J. Cell Biol. *110*, 903–914.

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

Heintz, N. H., and Stillman, B. W. (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.

Hendrickson, E. A., Fritze, C. E., Folk, W. R., and DePamphilis, M. L. (1987a). The origin of bidirectional DNA replication in polyoma virus. EMBO J. *6*, 2011–2018.

Hendrickson, E. A., Fritze, C. E., Folk, W. R., and DePamphilis, M. L. (1987b). Polyoma virus DNA replication is semi-discontinuous. Nucl. Acids. Res. *15*, 6369–6385.

Howarth, A. J., Gardner, R. C., Messing, J., and Shepherd, R. J. (1981). Nucleotide sequence of naturally occurring deletion mutants of cauliflower mosaic virus. Virology *112*, 678–685.

Hunter, T., Francke, B., and Bacheler, L. (1977). In vitro polyoma DNA synthesis: asymmetry of short DNA chains. Cell *12*, 1021–1028.

Iguchi-Ariga, S. M. M., Itani, T., Kiji, Y., and Ariga, H. (1987). Possible function of the c-myc product: promotion of cellular DNA replication. EMBO J. *6*, 2365–2371.

Iguchi-Ariga, S. M. M., Okazaki, T., Itani, T., Ogata, M., Sato, Y., and Ariga, H. (1988a). An initiation site of DNA replication with transcriptional enhancer activity present upstream of the c-myc gene. EMBO J. *7*, 3135–3142.

Iguchi-Ariga, S. M. M., Okazaki, T., Itani, T., and Ariga, H. (1988b). Cloning of the p53-dependent origin of cellular DNA replication. Oncogene *3*, 509–515.

Kaufmann, G., Bar-Shavit, R., and DePamphilis, M. L. (1978). Okazaki pieces grow opposite to the replication fork direction during simian virus 40 replication. Nucl. Acids Res. *5*, 2535–2545.

Kipling, D., and Kearsey, S. (1990). Reversion of autonomously replicating sequence mutations in Saccharomyces cerevisiae: creation of a eucaryotic replication origin within procaryotic vector DNA. Mol. Cell. Biol. *10*, 265–272.

Kohara, Y., Tohdoh, N., Jiang, X.-W., and Okazaki, T. (1985). The distribution and properties of RNA primed initiation sites of DNA synthesis at the replication origin of Escherichia coli chromosome. Nucl. Acids Res. *13*, 6847–6866.

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

Laskey, R. A., Fairman, M. P., and Blow, J. J. (1989). S phase of the cell cycle. Science *246*, 609–614.

Leu, T.-H., and Hamlin, J. L. (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.

Li, J. J., and Kelly, T. J. (1985). Simian virus 40 DNA replication in vitro: specificity of initiation and evidence for bidirectional replication. Mol. Cell. Biol. *5*, 1238–1246.

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

Ma, C., Leu, T.-H., and Hamlin, J. L. (1990). Multiple origins of replication in the dihydrofolate reductase amplicons of a methotrexate resistant Chinese hamster cell line. Mol. Cell. Biol. *10*, 1338–1346.

Mahmoudi, M., and Lin, V. K. (1989). Comparison of two different hybridization systems in northern transfer analysis. Biotechniques *7*, 331–333.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

Martin, R. G., and Setlow, V. P. (1980). The initiation of SV40 DNA synthesis is not unique to the replication origin. Cell *20*, 381–391.

McWhinney, C., and Leffak, M. (1990). Autonomous replication of a

DNA fragment containing the chromosomal replication origin of the human c-myc gene. Nucl. Acids Res. *18*, 1233–1242.

Micheli, G., Baldari, C. T., Carri, M. T., Cello, G. D., and Buongiorno-Nardelli, M. (1982). An electron microscope study of chromosomal DNA replication in different eukaryotic systems. Exp. Cell Res. *137*, 127–140.

Newlon, C. S. (1988). Yeast chromosome replication and segregation. Microbiol. Rev. *52*, 568–601.

Peden, K. W. C., Pipas, J. M., Pearson-White, S., and Nathans, D. (1980). Isolation of mutants of an animal virus in bacteria. Science *209*, 1392–1396.

Perlman, D., and Huberman, J. A. (1977). Asymmetric Okazaki piece synthesis during replication of simian virus 40 DNA in vivo. Cell *12*, 1029–1043.

Rabkin, S. D., and Richardson, C. C. (1988). Initiation of DNA replication at cloned origins of bacteriophage T7. J. Mol. Biol. *204*, 903–916.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual (Second Edition) (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

Seufert, W., and Messer, W. (1987). Start sites for bidirectional in vitro DNA replication inside the replication origin, oriC, of Escherichia coli. EMBO J. *6*, 2469–2472.

Stillman, B. (1989). Initiation of eukaryotic DNA replication in vitro. Annu. Rev. Cell Biol. *5*, 197–245.

Tack, L. C., and Proctor, G. N. (1987). Two major replicating SV40 chromosome classes: synchronous replication fork movement is associated with bound large T antigen during elongation. J. Biol. Chem. *262*, 6339–6349.

Tseng, B. Y., and Goulian, M. (1975). DNA synthesis in human lymphocytes; intermediates in DNA synthesis, in vitro and in vivo. J. Mol. Biol. *99*, 317–337.

Tsurimoto, T., and Matsubara, K. (1984). Multiple initiation sites of DNA replication flanking the origin region of λdv genome. Proc. Natl. Acad. Sci. USA *81*, 7402–7406.

Umek, R. M., Linskens, M. H. K., Kowalski, D., and Huberman, J. A. (1989). New beginnings in studies of eukaryotic DNA replication origins. Biochim. Biophys. Acta *1007*, 1–14.

Vassilev, L., and Russev, G. (1988). Purification of nascent DNA chains by immunoprecipitation with anti-BrdU antibodies. Nucl. Acids Res. *16*, 10397.

Vassilev, L. T., Burhans, W. C., and DePamphilis, M. L. (1990). Mapping an origin of DNA replication at a single copy locus in mammalian cells proliferating under physiological conditions. Mol. Cell. Biol., in press.

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

Vindelov, L. L., Christensen, I., and Nissen, N. (1983). A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. Cytometry *3*, 323–327.

Wigler, M., Pellicer, A., Silverstein, S., and Axel, R. (1978). Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as donor. Cell *14*, 725–731.