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## Research Summary

### Review of proposal relevance

The initiation of DNA replication in eukaryotic chromosomes represents one of the crucial events in the control of the cell division cycle. It is believed that this control is exerted primarily at the initiation of DNA replication at the thousands of origins spread across the genome. Each of the roughly 50,000 to 100,000 origins must be tightly regulated to maintain the integrity of the genome and to ensure that replication at each origin occurs only once per cell cycle. During normal development in some organisms, multiple rounds of replication at the same origin lead to an amplification of specific genomic sequences (Carminati et al. 1996, Breur et al. 1955, Liang et al. 1993, Spradling et al. 1999 and referenced therein). Under certain conditions, such as mutations in the p53 tumor suppressor gene, amplification can also occur in the human genome, and has been implicated as a prognostic factor in a number of cancers, including breast cancer (Slamon et al. 1987, Wright et al. 1989, Bonilla et al. 1988, Zhou et al. 1988, McWhinney and Leffak 1990, Vassilev and Johnson 1990, Tlsty et al. 1989, Tlsty 1990, Livingstone et al. 1992, Yin et al. 1992). Given that some amplification can be the result of overcoming the block to rereplication at origins of replication, it is imperative to understand the control of replication initiation in mammalian chromosomes in order to gain a better understanding of the role of amplification in breast cancer development and progression. Once the cis-acting sequences involved in DNA replication initiation are understood, their interaction with proteins that regulate replication initiation and amplification can be investigated.

One of the major gaps in our understanding of mammalian DNA replication is a genetic definition of a chromosomal origin of DNA replication. Initiation of DNA replication in *Escherichia coli*, mammalian viruses, and the budding yeast *Saccharomyces cerevisiae* (DePamphilis 1996, Newlon 1996) is controlled primarily by trans-acting initiator proteins that interact with a cis-acting DNA sequence element (the replicator) (Jacob et al. 1963). Since many of the initiator proteins found in yeast appear to be conserved in higher eukaryotes (reviewed in Dutta and Bell. 1997), it is possible that DNA replication initiation in mammalian cells may also be directed by a replicator and occur site-specifically within an initiation region (IR). Extensive mapping of replication start sites in mammalian chromosomes has revealed that replication at most, but not all, loci begins at a few high frequency start sites contained within a broad zone of initiation (reviewed in Gilbert 1998, DePamphilis 1999, Spradling 1999, Bogan et al. 2000, Toledo et al. 1998). One of the most thoroughly mapped high frequency initiation regions (IRs) in mammalian chromosomes is the region downstream from the dihydrofolate reductase (DHFR) gene in Chinese Hamster Ovary (CHO) cells (Fig. 1A). This region contains a 55 kb zone of delocalized origin activity containing three preferred

start sites: ori- $\beta$  centered approximately 17 kb downstream from the DHFR gene, ori- $\beta'$  just downstream from ori- $\beta$ , and ori- $\gamma$  located 23 kb further downstream (Heintz and Hamlin 1982, Heintz et al. 1983, Anachkova and Hamlin 1989, Handeli et al. 1989, Leu and Hamlin 1989, Burhans et al. 1990, Vassilev et al. 1990, Vaughn et al. 1990, Pelizon et al. 1996, Kobayashi et al 1998, Wang et al. 1998 Li et al. 2000).

Despite the progress in mapping initiation sites, the specific genetic elements necessary to direct initiation of mammalian DNA replication remain poorly understood. The existence of preferred IRs raises the question of whether specific DNA sequences within or neighboring an IR may direct initiation of replication at the IR or even in a broad zone more distant from the preferred IR. To address the question of whether initiation of DNA replication can be directed by specific sequences flanking the single preferred IR ori- $\beta$  of the DHFR locus, we have stably transfected an exogenous DNA fragment containing the ori- $\beta$  IR in random ectopic chromosomal locations and assayed for initiation activity by using a competitive polymerase chain reaction (PCR)-based nascent strand abundance assay.

### **Research summary**

The main goal of the research proposal was to develop an assay system for studying the specific genetic elements, if any, involved in the initiation of DNA replication in mammalian cells as outlined in Task 1. Since submission of the grant proposal, I have successfully developed a suitable assay for elucidating specific cis-acting elements involved in replication initiation (See attached manuscript, Altman and Fanning 2000). Briefly, a competitive polymerase chain reaction-based nascent strand abundance assay was used to demonstrate the ability of a small 5.7 kb fragment of DNA, containing the DHFR ori- $\beta$  initiation region, to support efficient origin activity when integrated into random ectopic positions in the hamster chromosome. In addition, the integrated ori- $\beta$  IR functioned with the same efficiency as the endogenous ori- $\beta$  IR in CHOK1 cells, suggesting that the 5.7 kb fragment was an acceptable candidate for mutational analysis of the ori- $\beta$  IR. In the early stages of developing the assay system, there were problems with the reproducibility of the data from the exogenous DHFR ori- $\beta$  fragments. For this reason, I developed a normalization method which facilitated the comparison of data from separate transfection experiments (See attached manuscript). This method greatly increased the reproducibility of the data from experiment to experiment, as well as providing a means of comparison between results from different deletion constructs (see below). Thus, Task 1 (development of assay system) has been successfully completed.

Studies of model systems such as animal viruses (SV40, Epstein-Barr virus, herpes simplex virus), mitochondria (human, mouse), protozoa (*Tetrahymena*) and yeast (*S.cerevisiae*, *Schizosaccharomyces pombe*), have determined that origins have a modular organization composed of unique DNA sequence motifs and interactions with soluble

proteins (reviewed in DePamphilis, 1993). Several regions of the 5.7 kb DHFR ori- $\beta$  fragment show sequence similarity to modular elements found in characterized origins. In order to determine whether these regions play a role in initiation of DNA replication in a mammalian origin, these regions were targeted for deletion analysis in the 5.7 kb DHFR ori- $\beta$  fragment. As seen in the attached manuscript, deletion of three of these putative elements led to a significant decrease in initiation activity, as assayed by the PCR-based nascent strand abundance assay. This suggests that, indeed, there are specific genetic elements that are necessary for efficient initiation of DNA replication in a mammalian origin. A fourth deletion did not decrease initiation activity, and thus was dispensable for origin activity. This result is important in that it demonstrates that not all assaults to the DHFR ori- $\beta$  fragment lead to a decrease in initiation activity. These studies successfully address Task 4 (identification of essential cis-acting sequences) in the statement of work and indicate that future analysis is worthwhile.

Development of the assay system was more difficult than expected given the problems of reproducibility, and the subsequent development of a normalization protocol. For this reason, significant work toward Task 2 (affect of linked resistance gene), Task 3 (assay ori- $\beta$  in human cancer cell lines) or Task 5 (test mutants in endogenous locus) has not yet been accomplished. However, now that the assay system is reliable, the execution of task 2 shall be rather simple. Task 3 may require the development of new PCR primers depending on the specificity of the established primer sets in the human cell lines, however, I do not foresee any other difficulties. Task 5 is a difficult and time-consuming process. It will require the development of a Cre recombinase system to target the DHFR ori- $\beta$  mutants to a specified chromosomal location. Recombination efficiency is not high and selection of the proper transformants is time-consuming, although these methods are currently being developed in the lab. For these reasons, Tasks 1-4 have taken precedence.

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## APPENDIX

### Key Research Accomplishments

- Development of a PCR-based nascent strand abundance assay for determining the initiation activity of exogenous DNA fragments containing the DHFR ori- $\beta$  IR.
- Development of normalization methods to permit comparison between independent transfection experiments.
- Demonstration that a 5.7 kb fragment containing the DHFR ori- $\beta$  IR is sufficient to direct initiation of DNA replication in the locus.
- Identification of three specific cis-acting sequence elements required for efficient initiation of DNA replication from DHFR ori- $\beta$ .
- Identification of one specific cis-acting sequence element that is dispensable for efficient initiation of DNA replication from DHFR ori- $\beta$ .

## **Reportable Outcomes**

### **Abstracts**

The Salk Institute Eukaryotic DNA Replication meeting, The Salk Institute, La Jolla, CA.  
September, 2000.

ORAL PRESENTATION "The Chinese hamster DHFR replication origin beta is active at multiple ectopic chromosomal locations and requires specific DNA sequence elements for activity."

Cold Spring Harbor Eukaryotic DNA Replication meeting, Cold Spring Harbor, NY.  
September, 1999.

POSTER: "Specific sequences are required to direct initiation of DNA replication in mammalian chromosomes."

Penn State's 18th Summer Symposium in Molecular Biology: Chromatin Structure and DNA Function. Penn State, State College, PA. July 1999.

POSTER: "Specific DNA sequences are required to direct initiation of DNA replication in mammalian chromosomes."

### **Publications**

Altman, A. L. and E. Fanning. 2000. The Chinese hamster DHFR replication origin beta is active at multiple ectopic chromosomal locations and requires specific DNA sequence elements for activity. *Mol. Cell. Biol.* submitted

The Salk Institute Eukaryotic DNA Replication meeting, The Salk Institute  
La Jolla, September, 2000.

**THE CHINESE HAMSTER DHFR REPLICATION ORIGIN BETA IS ACTIVE AT MULTIPLE ECTOPIC CHROMOSOMAL LOCATIONS AND REQUIRES SPECIFIC DNA SEQUENCE ELEMENTS FOR ACTIVITY.** Amy L. Altman and Ellen Fanning, Department of Biological Sciences and the Vanderbilt-Ingram Cancer Center, Vanderbilt University, Nashville, TN 37232

Mapping of replication start sites in mammalian chromosomes has revealed that initiation at most loci begins at a few high frequency start sites contained within a broad zone of initiation. One of the most thoroughly mapped high frequency initiation regions (IRs) in mammalian chromosomes is the region downstream from the dihydrofolate reductase (DHFR) gene in Chinese Hamster Ovary (CHO) cells. This region contains a 55 kb zone of less frequent start sites and three preferred start sites. Ori- $\beta$  is centered approximately 17 kb downstream from the DHFR gene, ori- $\beta'$  is just downstream from ori- $\beta$ , and ori- $\gamma$  is located 23 kb further downstream.

Despite the progress in mapping mammalian IRs, the specific genetic elements necessary to direct initiation of mammalian DNA replication remain poorly understood. To identify the elements essential for DNA replication initiation at the DHFR ori- $\beta$  preferred IR in mammalian chromosomes, we have stably transfected a 5.7 kb fragment of the DHFR locus containing the ori- $\beta$  initiation region into random ectopic chromosomal locations in a hamster cell line lacking the endogenous DHFR locus. Using a competitive PCR-based nascent strand abundance assay, initiation at ectopic ori- $\beta$  in pools of transfected cells and in six cloned cell lines was shown to mimic, qualitatively and quantitatively, initiation at the endogenous ori- $\beta$  in CHOK1 hamster cells. To assess the importance of specific DNA sequences for initiation activity, a panel of ori- $\beta$  deletion mutants was constructed and assayed in the same manner. A striking decrease in initiation activity, more than 10-fold, resulted when an extensive GA dinucleotide repeat was deleted from the distal end of the fragment. Deletion of an AT-rich region also reduced initiation activity to near background levels. Deletion of 4 bp between two AT-rich regions also led to a modest decrease in initiation activity. These results suggest (1) that a 5.7 kb fragment of DHFR ori- $\beta$  is sufficient to direct initiation at ectopic chromosomal locations, (2) that within this fragment, at least two widely spaced elements of very different sequence composition are required for efficient initiation activity, and (3) that the DHFR initiation zone may be composed of multiple independent replicators. (Supported by NIH GM 52948 and CA 09385, the Army Breast Cancer Program BC980907, and Vanderbilt University.)

Cold Spring Harbor Eukaryotic DNA Replication meeting, Cold Spring Harbor, NY.  
September, 1999.

**SPECIFIC DNA SEQUENCES ARE REQUIRED TO DIRECT  
INITIATION OF DNA REPLICATION IN MAMMALIAN  
CHROMOSOMES**

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The initiation of DNA replication represents one of the crucial control points in the eukaryotic cell division cycle. Although much progress has been made in the biochemical identification of preferred replication start sites in mammalian chromosomes, the specific genetic elements required for the initiation of DNA replication remain unclear. To begin to elucidate the genetic elements essential for DNA replication initiation *in vivo*, a 5.7 kb fragment of the hamster DHFR ori- $\beta$  preferred initiation region was stably transfected into random ectopic chromosomal locations in a CHO cell line which lacked any endogenous ori- $\beta$  DNA. Using competitive PCR to measure the abundance of specific exogenous DNA sequences in nascent DNA isolated from asynchronous cell pools, we demonstrated enhanced abundance of nascent DNA centered over the preferred start site that had been mapped at the endogenous locus in CHOK1 cells. The initiation efficiency in the transfected cell pools was comparable to or greater than that of the endogenous ori- $\beta$  in CHOK1 cells. Nascent DNA from cells transfected with mutant exogenous ori- $\beta$  fragments demonstrated a requirement for specific DNA sequences for initiation activity. Deletion of 4 bp centered within the preferred start site reproducibly decreased initiation activity to a level similar to that in nonreplicating CHOK1 cells. Deletion of an A-T rich element, which shares sequence homology with a putative human lamin B2 origin protein binding site (Abdurashidova et al., 1998. EMBO J. 17, 2961-2969), resulted in a similar reproducible decrease in initiation activity. In contrast, large deletions of the 3' end of the DHFR ori- $\beta$  fragment caused great variation in initiation activity, suggesting that this region is required for reproducible efficient initiation of DNA replication at ori- $\beta$  in ectopic sites and may represent an element which either enhances initiation activity or insulates the putative origin from local chromosomal context. Taken together, these data suggest that the 5.7 kb fragment is sufficient to direct efficient, reproducible initiation of DNA replication *in vivo* at DHFR ori- $\beta$  in ectopic sites, and that this fragment contains several specific genetic elements which are necessary for this initiation.

Penn State's 18th Summer Symposium in Molecular Biology: Chromatin Structure and DNA Function. Penn State, State College, PA. July 1999.

**SPECIFIC DNA SEQUENCES ARE REQUIRED TO DIRECT INITIATION OF DNA REPLICATION IN MAMMALIAN CHROMOSOMES;** A.L. Altman and E. Fanning, Department of Molecular Biology and the Vanderbilt-Ingram Cancer Center, Vanderbilt University, Nashville, TN 37232

The initiation of DNA replication represents one of the crucial control points in the eukaryotic cell division cycle. Although much progress has been made in the biochemical identification of preferred replication start sites in mammalian chromosomes, the specific genetic elements required for the initiation of DNA replication remain unclear. To begin to elucidate the genetic elements essential for DNA replication initiation *in vivo*, a 5.7 kb fragment of the hamster DHFR ori- $\beta$  preferred initiation region was stably transfected into random ectopic chromosomal locations in a CHO cell line which lacked any endogenous ori- $\beta$  DNA. Using competitive PCR to measure the abundance of specific exogenous DNA sequences in nascent DNA isolated from asynchronous cell pools, we demonstrated enhanced abundance of nascent DNA centered over the preferred start site that had been mapped at the endogenous locus in CHOK1 cells. The initiation efficiency in the transfected cell pools was comparable to or greater than that of the endogenous ori- $\beta$  in CHOK1 cells. Nascent DNA from cells transfected with mutant exogenous ori- $\beta$  fragments demonstrated a requirement for specific DNA sequences for initiation activity. Deletion of 4 bp centered within the preferred start site reproducibly decreased initiation activity to a level similar to that in nonreplicating CHOK1 cells. Deletion of an A-T rich element, which shares sequence homology with a putative human lamin B2 origin protein binding site (Abdurashidova et al., 1998. *EMBO J.* 17, 2961-2969), resulted in a similar reproducible decrease in initiation activity. In contrast, large deletions of the 3' end of the DHFR ori- $\beta$  fragment caused great variation in initiation activity, suggesting that this region is required for reproducible efficient initiation of DNA replication at ori- $\beta$  in ectopic sites and may represent an element which either enhances initiation activity or insulates the putative origin from local chromosomal context. Taken together, these data suggest that the 5.7 kb fragment is sufficient to direct efficient, reproducible initiation of DNA replication *in vivo* at DHFR ori- $\beta$  in ectopic sites, and that this fragment contains several specific genetic elements which are necessary for this initiation.

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**The Chinese hamster DHFR replication origin beta is active at multiple ectopic chromosomal locations and requires specific DNA sequence elements for activity**

Running Title: ori- $\beta$  requires specific cis-acting sequence elements

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**ABSTRACT**

To identify cis-acting genetic elements essential for mammalian chromosomal DNA replication, a 5.7 kb fragment from the Chinese hamster dihydrofolate reductase (DHFR) locus containing the ori- $\beta$  initiation region was stably transfected into random ectopic chromosomal locations in a hamster cell line lacking the endogenous DHFR locus. Initiation at ectopic ori- $\beta$  in uncloned pools of transfected cells was measured using a competitive PCR-based nascent strand abundance assay and shown to mimic that at the endogenous ori- $\beta$  in CHOK1 hamster cells. Initiation activity of three ectopic ori- $\beta$  deletion mutants was reduced, while the activity of another deletion mutant was enhanced. The results suggest that a 5.7 kb fragment of DHFR ori- $\beta$  is sufficient to direct initiation and that specific DNA sequences in ori- $\beta$  are required for efficient initiation activity.

Key Words: DHFR ori- $\beta$  / DNA replication/ replicator elements/ origin of replication

## INTRODUCTION

Initiation of DNA replication in *Escherichia coli*, mammalian viruses, and the budding yeast *Saccharomyces cerevisiae* (23, 67) is controlled primarily by trans-acting initiator proteins that interact with a cis-acting DNA sequence element (the replicator) (46). In these simple replicons, the cis-acting element consists of an essential core sequence, containing initiator protein binding sites and easily unwound sequences (DNA unwinding elements), and auxiliary sequences which enhance the efficiency of replication initiation (23, 67). In budding yeast, the initiation of replication requires an autonomously replicating sequence (ARS) element in cis and occurs within a region flanked by a DNA unwinding element and the binding sites for the origin recognition complex (ORC) and other initiator proteins (10). The initiation site for bidirectional replication appears to be restricted to a single nucleotide in a chromosomal ARS element (11). Replication initiation in fission yeast is also controlled by the sequence-specific recognition of a cis-acting replicator by ORC and associated initiator proteins, but the fission yeast replicators are larger than those of its budding yeast counterpart (50, 19, 69, 68, 66, 18). Initiation activity of yeast ARS elements in chromosomal DNA depends not only on specific DNA sequences in the ARS, but also on chromatin structure and chromosomal position (79, 13, 67, 70, 34, 36, 88, 51, 62).

Extensive mapping of replication start sites in mammalian chromosomes has revealed that replication at most, but not all, loci begins at a few high frequency start sites contained within a broad zone of initiation (83, 63, 82, 37, 4, 59, and other studies reviewed in 40, 24, 12). One of the most thoroughly mapped high frequency initiation

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regions (IRs) in mammalian chromosomes is the region downstream from the dihydrofolate reductase (DHFR) gene in Chinese hamster ovary (CHO) cells (Fig. 1A). This region contains a 55 kb zone of delocalized origin activity containing three preferred start sites: ori- $\beta$  centered approximately 17 kb downstream from the DHFR gene, ori- $\beta'$  just downstream from ori- $\beta$ , and ori- $\gamma$  located 23 kb further downstream (43, 44, 5, 42, 55, 14, 85, 86, 71, 52, 89, 57).

Despite the progress in mapping initiation sites, the specific genetic elements necessary to direct initiation of mammalian DNA replication remain poorly understood. The existence of preferred IRs raises the question of whether specific DNA sequences within or neighboring an IR may direct initiation of replication at the IR or even in a broad zone more distant from the preferred IR. Several lines of evidence are consistent with the notion that sequences neighboring a preferred IR may constitute a mammalian replicator element. In the human lamin B2 IR, DNase I footprinting has revealed that a specific sequence is protected in a cell cycle-dependent manner, suggesting that it may be a binding site for an initiator protein complex (39, 29, 1). Moreover, replication at the lamin B2 origin was shown to initiate within a three base pair sequence that overlaps the footprint region (2). A site hypersensitive to micrococcal nuclease has been mapped in the DHFR ori- $\beta$  IR locus (72) and there is preliminary evidence that hamster ORC2, a subunit of the ORC complex, binds within the DHFR ori- $\beta$  IR (cited in 12). Recent genetic analysis of the human  $\beta$ -globin (4) and the human c-myc (63) initiation regions at an ectopic chromosomal locus demonstrated that a defined sequence of 2 to 8 kilobases can be sufficient to direct initiation in the ectopic locus. Furthermore, the putative c-myc replicator even induced new start sites in the flanking chromosomal DNA (63). Taken

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together, these studies suggest that initiation of chromosomal DNA replication in mammalian cells may be directed by specific cis-acting DNA sequence elements that constitute a replicator similar to those characterized in yeast.

The complex organization of the DHFR initiation zone raises the question of whether it differs in principle from the putative replicators containing the c-myc, lamin B2, and  $\beta$ -globin IRs. For example, the cluster of preferred IRs may represent a novel replicator organization with multiple elements dispersed throughout the 55 kb initiation zone that are interdependent and cannot function independently as replicators. Alternatively, the three preferred start sites ori- $\beta$ , ori- $\beta'$  and ori- $\gamma$  within the delocalized initiation zone could represent separable, but redundant genetic elements that ensure the faithful replication of this locus (72). In this latter case, the region surrounding each of the three start sites might serve as an individual replicator able to direct initiation when placed at an ectopic chromosomal location.

To address the question of whether a defined DNA sequence encompassing DHFR ori- $\beta$  can direct initiation of DNA replication in chromosomal DNA, we have generated stably transfected cells containing the exogenous ori- $\beta$  IR in random ectopic chromosomal locations and measured ori- $\beta$  initiation activity by using a competitive polymerase chain reaction (PCR)-based nascent strand abundance assay. The results presented here demonstrate that a 5.7 kb sequence, containing the ori- $\beta$  IR, is sufficient to direct initiation in multiple ectopic chromosomal sites, and that specific DNA sequences within and flanking the IR are essential for efficient initiation activity at the DHFR ori- $\beta$ .

## MATERIALS AND METHODS

**Cell culture.** Diploid Chinese hamster ovary (CHOK1) cells and DR12 cells, a CHOK1 derivative containing a 150 kb deletion of the entire DHFR locus (47), were grown in Ham's F12 media supplemented with 10% fetal calf serum (Life Technologies, Rockville, MD) at 37° C and 4% CO<sub>2</sub>.

**Plasmid constructs.** The plasmid pMCD, containing the 5.7 kb Bam HI-Kpn I fragment, nucleotides 1-5793 (Genbank Acc. No. Y09885), of the DHFR ori- $\beta$  locus in the vector pUC19 (90), was the kind gift of Nick Heintz (16). Deletion mutant pAKO was generated by digestion of pMCD with Apa I (nt position 1127), removal of overhanging ends using T4 DNA polymerase, and religation. Mutant pMCD $\Delta$ AT was created by partial digestion of pMCD with Sph I (nt position 2163), followed by complete digestion with Eco RV (nt position 2507), releasing a 344 bp fragment; the other fragment was then blunt-ended and religated. The mutant pMCD $\Delta$ DNR was created by partial digestion of pMCD with Xba I (nt position 4454) followed by complete digestion with Nhe I (nt position 4219), releasing a 235 bp fragment; the remaining fragment was blunt-ended and religated. Mutant pMCD $\Delta$ TR was created by using PCR and mutagenic primers (5'GACAAAAACAATCGATAAATAAG, 5'CTTATTTATCGATTGTTT TTGTC) to insert a Cla I restriction site at nucleotide position 686, relative to the Bam HI site. The resulting plasmid was partially digested with Pvu II (nt position 949), followed by complete digestion with Cla I, releasing a 263 bp fragment; the remaining fragment was blunt-ended and religated. The pSV2neo plasmid contains a full-length

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neomycin resistance gene (Clontech Laboratories, Inc., Palo Alto, CA). Plasmid KI26 contains the 3 kb Bam HI-Xba I fragment of the DHFR ori- $\beta$  region in the vector pBluescript. Plasmid Mut8-2 contains the Sac I/ Acc I fragment of the DHFR ori- $\beta$  region in the vector pBluescript.

**Transfection.** The Bam HI-Kpn I ori- $\beta$  fragment cloned in pUC19 was linearized with Ava II to generate the 5.7 kb DHFR insert flanked by 1271 and 1401 bp of vector DNA. Four  $\mu$ g of the digest mixed in a 3:1 molar ratio with Pvu I-linearized pSV2neo DNA was electroporated into  $5 \times 10^6$  DR12 cells using a BioRad Gene Pulser at 360 V and 600  $\mu$ F. Cells were plated in 75 cm<sup>2</sup> tissue culture flasks. After 24 h, the medium was replaced with Ham's F12 supplemented with 10% FCS and 0.5 mg/ml G418 (Life Technologies, Rockville, MD). After 4 weeks of growth under selection, exponentially proliferating cells from four 150 cm<sup>2</sup> flasks grown to 70% confluency were harvested and total DNA was isolated and analyzed as described below. Under these transfection conditions, the average number of copies of exogenous DHFR ori- $\beta$  integrated per cell in pools of cells was highly reproducible (see below). Single cell clones were isolated through serial dilution into a 96 well plate and expanded under drug selection for 14 weeks to four 150 cm<sup>2</sup> flasks. Isolation of total genomic DNA and analysis were carried out as with pools of transfected cells.

**Quantitation of stably transfected DNA.** The integration of ectopic DHFR ori- $\beta$  fragments into DR12 genomic DNA was monitored by PCR analysis of DNA from transfected cells. PCR amplification was carried out with primer set 2 and 5 ng of total

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genomic DNA, isolated from transfected or from CHOK1 cells, in a Perkin Elmer 4800 thermal cycler (94° C for 30 sec, 56° C for 30 sec, 72° C for 30 sec; 35 cycles).

Amplification products were resolved by 7% polyacrylamide gel electrophoresis, stained with ethidium bromide, and quantified by densitometry (IPLab Gel software, Signal Analytics Corporation, Vienna, VA). The ratio of the exogenous DHFR ori- $\beta$  products to the endogenous CHOK1 ori- $\beta$  products, termed integration ratio, was consistently close to 1 after 4 weeks of drug selection. In some experiments, the PCR analysis was carried out in parallel with primer pair 2 and with primer pair 3; the resulting ratios did not differ significantly.

**DNA isolation and gradient centrifugation.** Total genomic DNA was isolated from harvested cells according to the manufacturer's instructions (Nucleon II, Scotlabs). 500  $\mu$ g of resuspended total genomic DNA in 1 ml of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) was heat denatured at 100° C for 10 min, followed by a 10 min incubation in ice-water. DNA was loaded onto a 5-30% linear neutral sucrose gradient, and centrifuged at 26,000 rpm (Sorvall AH-629 rotor) for 17 h at 20° C. Fractions of 1 ml were collected and the fraction enriched in nascent single-stranded DNA ca. 1-2 kb in length was dialyzed against TE and used as a template for PCR amplification. As size markers, 50  $\mu$ g of pMUT8-2, digested with Kpn I and Nde I to produce fragment sizes of 921 bp and 2972 bp, and 50  $\mu$ g of pK126, digested with Stu I and Bgl II to produce fragment sizes of 1636 bp and 4338 bp were loaded on a sucrose gradient and run concurrently with each set of experiments. A 50  $\mu$ l aliquot of each marker fraction was subjected to electrophoresis on a 1% agarose gel at 200 mA for 2 hrs, and DNA was visualized by

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ethidium bromide staining. For control PCR reactions, total genomic DNA was sheared to fragments 1-2 kb in size by sonication for 15 sec at 25% power (Branson 250/450 Sonifier, Branson Ultrasonics Corp., Danbury, CT), heat-denatured, and fractionated on a 5-30% linear neutral sucrose gradient as described above.

**Construction of PCR competitive templates.** Competitors were constructed essentially as previously described (71). Briefly, PCR amplification was performed with pMCD template using the standard primer sets 8, 2, 6, and 3 and their corresponding competitor (COM) primer sets, which contained a 20 bp insertion (Table 1). These PCR products were used as templates for another round of PCR amplification with the standard primer sets, resulting in PCR amplification products which were identical to their pMCD target sequence except for the 20 bp insertion. The mutated PCR amplification products were cloned into pMCD to create competitive templates for the PCR-based nascent strand abundance assay.

**PCR-based nascent strand abundance assay.** The competitive PCR nascent strand abundance assay was performed using primer sets 8, 2, 6, and 3 (71, 31, 53; Table 1) essentially as previously described. Each PCR reaction included the corresponding competitor plasmid DNA, which had been pre-calibrated against 20 ng of total genomic DNA from asynchronously growing CHOK1 cells. Assuming  $3 \times 10^9$  bp per haploid genome, 20 ng of genomic DNA would correspond to 6000 molecules of competitor (31). Amplification reactions with each primer set contained increasing amounts of the size-fractionated nascent DNA and the pre-calibrated amount of the corresponding competitor

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DNA. PCR was carried out in a Perkin Elmer 4800 thermal cycler (94° C for 30 sec, 56° C for 30 sec, 72° C for 30 sec; 50 cycles). Similar results were obtained using 35 cycles with the same program (data not shown), consistent with the expectation that competitive PCR should be independent of cycle number (31). Amplification products were resolved by 7% polyacrylamide gel electrophoresis, stained with ethidium bromide, and quantified by densitometry (IPLab Gel software, Signal Analytics Corporation, Vienna, VA). The intensity of the signal was linearly proportional to the amount of stained DNA under the conditions used.

The ratio of PCR amplification products from nascent genomic template relative to the PCR amplification products from the competitive template (nascent/competitor) was plotted on the X-axis against the volume of input nascent genomic DNA template on the Y-axis. Linear regression analyses were performed on the data (KaleidaGraph software, Synergy Software, Reading, PA), resulting in correlation coefficients greater than 0.97. The slope of this linear regression, along with the known molecules/ $\mu$ l of calibrated competitor, was used to calculate the molecules/ $\mu$ l of nascent DNA for each primer set. Since the number of base pairs in each amplification product is known, the g/mole of target DNA can be calculated using the conversion of 660 g/mole bp. By converting the molecules/ $\mu$ l of nascent DNA target for each primer set to mole/ml using Avagadro's number, and multiplying this by the g/mole of target DNA, the DNA concentration of nascent DNA target for each primer pair can be calculated. The concentration of target molecules for each primer pair can then be expressed relative to the concentration of total DNA in the nascent fraction, as determined by absorbance at 260 nm (termed abundance). To facilitate comparison between separate experiments, the

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abundance of target sequences for each primer pair was expressed as a ratio to the abundance of the target sequences for the distal pp3 in the same experiment (termed initiation activity).

## RESULTS

**A DHFR ori- $\beta$  fragment functions efficiently as an origin in ectopic chromosomal locations.** Given that DNA fragments containing the  $\beta$ -globin (4) and the c-myc (63) initiation regions (IRs) supported efficient DNA replication at ectopic chromosomal locations, we asked whether a small DNA fragment containing DHFR ori- $\beta$  and flanking DNA, but lacking the downstream ori- $\beta'$  and ori- $\gamma$ , would direct initiation of DNA replication when located at ectopic chromosomal sites. To address this question, we chose the experimental strategy diagramed in Fig. 1B. A 5.7 kb fragment containing the ori- $\beta$  IR (pMCD, 16), but not ori- $\beta'$ , ori- $\gamma$ , matrix attachment regions, or DHFR coding sequences, was cotransfected by electroporation with an unlinked neomycin resistance marker into DR12 cells, a CHOK1-derived line lacking both DHFR loci (47). We chose an unlinked resistance marker to ensure that any initiation of DNA replication occurring within the ori- $\beta$  fragment would not be influenced by transcription of the neomycin gene in the same construct. To facilitate the stable integration of the DNA fragments into the chromosomal DNA, plasmids containing the ori- $\beta$  DNA fragment were first linearized in the vector portion by restriction endonuclease digestion. Under these conditions, integration of exogenous DNA fragments has been reported to be random, and without significant loss of DNA sequences at the ends of the fragment (77, 35). As a strategy to

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minimize potential position effects of the integration site, nascent DNA was isolated from pools of uncloned transfectants. We reasoned that if position effects caused by flanking DNA at the integration sites of the ectopic chromosomal ori- $\beta$  fragments occurred, they might be masked in pools of uncloned cells, thereby enhancing the reproducibility of the assay. Total DNA was heat-denatured, and size-fractionated by density gradient centrifugation. The fraction containing single-stranded DNA ca. 1-2 kb in length was used as a template for PCR amplification in competitive PCR-based nascent strand abundance assays with four sets of primers (Fig. 1A). Three of the selected primer sets (pp8, pp2, pp6) were located within and directly flanking the IR, as determined by previous mapping studies (71, 52, 57). A fourth primer set (pp3), located distal to the IR and outside of the 1-2 kb nascent DNA strand template, was used to normalize the data to an outlying non-origin primer set, and facilitate comparison between separate experiments. Hence, the initiation activity of the ectopic IR was expressed as the ratio of the IR target sequences over the non-IR sequence in the 1-2 kb single-stranded DNA fraction.

To first validate our competitive PCR-based nascent strand abundance assay, it was used to confirm the preferential initiation at the endogenous DHFR ori- $\beta$  in CHOK1 cells. DNA sequences that are close to ori- $\beta$  are expected to be enriched in the short single-stranded DNA fraction, compared to more distal DNA sequences which are expected to be represented only in longer nascent DNA strands. Consistent with previous studies (71, 52, 57), the amount of nascent DNA template required to amplify an amount of target DNA equivalent to the precalibrated competitor DNA with primer pair 2 (pp2), centered over the previously mapped ori- $\beta$  IR, was smaller than with primer pairs 8, 6,

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and 3, which were more distant from the IR (Fig. 2A). Quantitative evaluation of one such experiment is shown in Table 2. The abundance of pp2 target DNA sequences was more than 10-fold higher than the abundance of target sequences for the outlying pp3 in the nascent CHOK1 DNA fraction. To compare the abundance of pp2 target sequence in nascent CHOK1 DNA among multiple independent experiments, it was expressed relative to the abundance of pp3 sequences in each experiment to give initiation activity. The average initiation activity from five independent experiments with CHOK1 cells is shown in Fig. 2C (black bars). Consistent with previous (71, 52, 57), the initiation activity centered over the previously mapped IR in the endogenous DHFR ori- $\beta$  was strongly enhanced compared to that in the flanking sequences (Fig. 2C, black bars).

To establish a base line or background value for comparison with nascent strand abundance data, the experiment was repeated under identical conditions, except that total CHOK1 DNA, isolated from asynchronously growing cells, was sheared to 1-2 kb fragments, heat-denatured and size-fractionated. The fraction containing the sheared, single-stranded DNA of 1-2 kb in size was used as the template in competitive PCR assays. In total genomic DNA, the target sequences for each primer pair should be essentially equally represented and thus, the amounts of amplification product generated with each primer pair should be similar. Indeed, the amounts of amplified DNA were similar with three of the four primer pairs, and somewhat reduced for pp8 compared to the other primer pairs (Fig. 2B, Table 2). Comparison of these results with the enhanced abundance of pp2, pp6, and pp8 target sequences in the nascent DNA fraction from asynchronous CHOK1 cells indicated that the peak of initiation activity depended on the enrichment for nascent DNA template and did not arise through differences between

primers in amplification efficiency or calibration error (Fig. 2C, compare black and white bars). Thus, the abundance of target sequences generated by amplification of the sheared-DNA control was considered to represent the empirical background activity of the assay system.

The PCR-based nascent strand abundance assay was then used to measure initiation activity at ectopic ori- $\beta$  sequences in genomic DNA from pools of stably transfected DR12 cells. The transfection conditions were chosen to ensure that the average copy number of ectopic ori- $\beta$  fragments per cell in the pool of cells was close to that of the endogenous ori- $\beta$  in CHOK1 DNA. To confirm this, total genomic DNA isolated from uncloned drug-resistant pools of DR12 cells was used as a template for PCR amplification. For comparison, an equal amount of total genomic DNA isolated from CHOK1 cells was used as a template in a parallel amplification. The amplification products of both reactions were visualized by PAGE and ethidium bromide staining. Amounts of amplification product obtained with both samples were quantitated and found to be very similar, implying that the average copy number of ori- $\beta$  per transfected cell in the pool was about the same as that of the endogenous ori- $\beta$  in CHOK1 cells (Fig. 3A).

The abundance of ori- $\beta$  target sequences in nascent DNA originating from the ectopic pMCD fragment in pools of transfected DR12 cells was then quantitated by competitive PCR. As seen in Fig. 3B, the amount of nascent DNA needed to amplify an amount of target DNA equivalent to the precalibrated competitor DNA with primer pair 2 was ten-fold less than with the flanking pp8 and the distal pp3, and several-fold less than with the flanking pp6. The abundance of target DNA for each primer pair in the

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nascent DNA fraction of the transfected cell pool was similar to that of the corresponding target DNA in nascent DNA from CHOK1 cells, as shown in the example in Table 2.

Combining data from seven independent transfection experiments revealed that the target sequence for primer pair 2 was significantly more abundant in the nascent DNA fraction than flanking target sequences, as would be expected for an active origin of DNA replication (Fig. 3C, black bars). The initiation activity of the ectopic ori- $\beta$  IR was enhanced more than 10-fold relative to that in the distal pp3 sequences, closely resembling the results obtained with endogenous ori- $\beta$  in CHOK1 cells (compare Fig. 3C and Fig. 2C). These results suggest that the 5.7 kb fragment of DHFR ori- $\beta$  is sufficient to direct initiation in ectopic chromosomal locations and that the ectopic ori- $\beta$  fragment functions with an efficiency comparable to that in the endogenous locus.

**Ori- $\beta$  functions in multiple ectopic locations, but exhibits some position effects.** Since chromosomal context is an important determinant of origin function in mammalian cells (17, 54, 56, 3, 27, 33, 30), the function of the exogenous ori- $\beta$  IR may be sensitive to position effects that were not detected in the uncloned cell pools. If initiation activity of the 5.7 kb fragment is affected by chromosomal context, one might expect to find that initiation activity of ectopic ori- $\beta$  would vary among clonal cell lines with different ori- $\beta$  integration sites. To test for variability in initiation activity, six individual cell lines were cloned from a resistant cell pool. To estimate the amount of integrated exogenous ori- $\beta$  fragment in each clone, total genomic DNA isolated from each cell line was used as a template for PCR amplification with pp2. For comparison with endogenous ori- $\beta$ , an equal amount of total genomic DNA isolated from CHOK1 cells

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was used as a template in a parallel amplification. The amplification of the exogenous ori- $\beta$  DNA fragments differed slightly from clone to clone. Clones 1, 3, and 5 contained about the same copy number found for the endogenous ori- $\beta$  in CHOK1 DNA (Table 3).

Clones 2, 4, and 6 had 20-30% less ori- $\beta$  fragment, possibly suggesting some loss of ori- $\beta$  sequences during expansion of the cloned cell lines.

The abundance of the exogenous ori- $\beta$  IR target sequences in the nascent DNA fraction was quantitated for each cell clone, as shown in the examples in Table 3. For each cell clone, the abundance of target DNA for pp2 was higher than the abundance of target DNA for the flanking primer sets (Table 3). The relative initiation activity of the clones varied from 2.7 to 9.4-fold higher than in the distal pp3 sequences. Thus, the initiation activity of the clones ranged from just over background in two of the clones to about half the activity observed with uncloned pools of cells (compare Table 3 and Fig. 3C). The strong activity observed for the pMCD-transfected cell pools in repeated experiments (Fig. 3C) suggests that the variability from one clone to another was probably masked when pools of transfected cells containing many different integration sites were tested. Although the ectopic ori- $\beta$  was less active in each of the clones than in a typical experiment with an uncloned pool of transfectants (compare Tables 2 and 3), these results suggest that the exogenous 5.7 kb ori- $\beta$  fragment was functional to some degree in six ectopic locations.

**Specific cis-acting sequences are required for efficient DHFR ori- $\beta$  initiation activity.** To assess whether specific DNA sequences within the 5.7 kb fragment of ori- $\beta$  were required for efficient initiation at the preferred IR, several ori- $\beta$

deletion mutants were constructed (Fig. 4A). The regions chosen for deletion contain DNA sequences that could be functionally important in mammalian origins based on their resemblance to DNA elements identified in other origins as discussed below. To permit comparison of the initiation activity of mutated fragments with that of the parental pMCD ori- $\beta$  fragment, mutations that would delete primer sites for PCR amplification were avoided.

The downstream end of the 5.7 kb DHFR fragment contains a region of GA dinucleotide repeats which has been shown to adopt non-B form DNA structures (16). Such *Drosophila* dinucleotide repeats have been postulated to slow or arrest replication fork progression in rodent cells (74, 7). To determine whether the GA repeat element affected ori- $\beta$  initiation activity, we created a construct which deleted the GA repeat (pMCD $\Delta$ DNR, Fig. 4A).

The 5.7 kb ori- $\beta$  DNA fragment contains a central AT-rich region (Fig. 4A) that was previously proposed to be a DNA unwinding element (16). This region shares sequence homology with a cell cycle-dependent protein binding site in the human lamin B2 IR (39, 29, 1), suggesting it as a candidate for an initiator protein binding site (Fig. 4A, hatched box). Moreover, the AT-rich region has homology to the recently reported ORC-binding sequences in chorion gene amplification control element ACE3 (6, 80) (Fig. 4A, checkered box). It also has homology with an AT-rich motif M found in *S. pombe* ARS elements (50). In order to determine whether the central AT-rich sequence in DHFR ori- $\beta$  contributes to initiation activity, we deleted a 344 bp region containing this element from the 5.7 kb fragment (pMCD $\Delta$ AT, Fig. 4A).

The DHFR ori- $\beta$  IR contains an AT-rich DNA sequence similar to ACE3 ORC binding sites (Fig. 4A, checkered box) (6, 80, 12). It also contains a stably bent DNA sequence and binding sites for the RIP60 protein (15, 21, 16, 64, 45). A deletion of 4 bp in a GC-hexanucleotide between these elements was constructed to explore the effects of a small deletion on initiation activity (Fig. 4A, pAKO). Such minimal mutations in ACS of budding yeast ARS elements have been shown to reduce or completely abolish ARS activity (84, 76, 58) by preventing ORC binding to the ARS (9, 75, 78, 32). A single base pair insertion between two T antigen binding sites in the SV40 origin has also been shown to destabilize T antigen binding by altering the spacing, and consequently the interactions, between the two T antigen hexamers on DNA (20, 87).

Finally, a region upstream of the DHFR ori- $\beta$  IR with an extensive T-rich stretch in one strand was chosen for deletion. Many characterized origins contain a sequence composed of a T-rich and an A-rich strand, the length of which appears to be critical for origin function, perhaps to facilitate strand separation (reviewed in 23). The T-rich region in ori- $\beta$  contains a prominent cell-cycle dependent nuclease hypersensitive site (72) (Fig. 4A, striped box) and an Alu repeat. Some Alu repeats have been correlated with DNA amplification and with autonomous replication activity of plasmid DNA (8, 65, 48). The T-rich region has sequence homology to the *Drosophila* ACE3 element (6, 80) (Fig. 4A, checkered box), and homology with AT-rich motifs C and D found in *S. pombe* ARS elements (50). In order to determine whether this T-rich region affects the initiation activity of the 5.7 kb fragment, we deleted a 263 bp region containing this element (pMCD $\Delta$ TR, Fig. 4A).

Mutant DNA fragments were transfected into DR12 cells and drug-resistant pools of cells were selected. The amount of integrated exogenous ori- $\beta$  fragment in each pool was monitored by PCR analysis of the genomic DNA as in Fig. 3A. Fig. 4B shows that the amount of exogenous ori- $\beta$  in DNA from the mutant-transfected cell pools was about the same as the endogenous ori- $\beta$  in an equal amount of CHOK1 DNA. Thus, the mutant origins were stably associated with chromosomal DNA in the same manner as the transfected wildtype 5.7 kb ori- $\beta$  fragment (compare Fig. 4B and Fig. 3A).

The abundance of ori- $\beta$  sequences in the small nascent DNA fraction prepared from pools of uncloned transfectants was then assayed by competitive PCR. A typical experiment with each mutant is shown in Fig. 4C and quantitatively evaluated in Table 4. The abundance of target DNA for pp2 in the nascent DNA fraction from three of the transfected mutants (pMCD $\Delta$ DNR, pMCD $\Delta$ AT, pAKO) was significantly lower than the abundance of pp2 target sequences in nascent DNA from transfected wildtype ori- $\beta$  (Table 4). Comparing the abundance of pp2 target sequence in nascent DNA with that of flanking target sequences demonstrates that the initiation activity of these three deletion mutants was markedly reduced. In contrast, deletion of the upstream T-rich element (pMCD $\Delta$ TR) resulted in a 4-fold increase in the abundance of pp2 target sequence relative to the abundance of the flanking pp3 target sequence (Table 4), indicating that deletion of the upstream T-rich element enhanced initiation activity at the ectopic ori- $\beta$  locus.

To facilitate comparison between the mutants, the abundance of target sequences for each primer pair in three separate transfection experiments with each mutant was normalized to the abundance of target sequences for pp3 in the same experiment. As seen

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in Fig. 4D, the initiation activity for pMCD $\Delta$ DNR-transfected cell pools was strongly and reproducibly reduced compared to pMCD-transfected cell pools (two-tailed t-test p-value: 0.01, representing a confidence of 98%). Initiation activity of pMCD $\Delta$ AT-transfected cell pools was also significantly reduced compared to wildtype-transfected pools (two-tailed t-test p-value: 0.02, representing a confidence of 96%). Indeed, the initiation activities with the pMCD $\Delta$ DNR and pMCD $\Delta$ AT mutants were only slightly above the empirical background for the assay as determined using sheared total DNA (Table 4, Fig. 3C, white boxes). Deletion of 4 bp within the IR (pAKO) significantly diminished the initiation activity; but the reduction was more modest (two-tailed t-test p value: 0.10, representing a confidence of 80%). Deletion of the upstream T-rich element (pMCD $\Delta$ TR) clearly stimulated initiation activity (two-tailed t-test p value: 0.01, representing a confidence of 98%) compared to the wildtype activity. The results demonstrate that the sequences deleted in three of the mutants were critical for ori- $\beta$  activity. In contrast, the T-rich region was dispensable for the initiation of DNA replication at ori- $\beta$ , and suggest that it may suppress initiation activity of the 5.7 kb fragment.

## DISCUSSION

**A 5.7 kb region surrounding the DHFR ori- $\beta$  functions as an independent chromosomal replicator.** Evidence presented in this study indicates that a 5.7 kb fragment of the DHFR ori- $\beta$  placed in random ectopic chromosomal locations was sufficient to direct initiation of DNA replication from the ori- $\beta$  start site (Fig. 3C). These

data were obtained by using a competitive PCR-based nascent strand abundance assay with nascent template DNA enriched only by heat-denaturation and size-selection. Thus, a possible concern is that the template fraction contained not only nascent DNA, but probably also small DNA fragments generated by shearing in vitro and single strand breaks in vivo. To validate the preparation methods and assays used to assess the initiation activity of the 5.7 kb ectopic ori- $\beta$  fragment, they were initially tested with the endogenous DHFR locus (Fig. 2C). Direct comparison of our data on nascent strand abundance at ori- $\beta$  in CHOK1 cells with data obtained by using lambda exonuclease digestion to further enrich for RNA-primed nascent DNA after the size-fractionation (38) reveals remarkable congruence (52). The ratio of nascent DNA at the center of the ori- $\beta$  IR to flanking non-replicating DNA in our study averaged about 15-fold in multiple experiments (Fig. 2C), quantitatively comparable to the 12-fold enrichment reported by DePamphilis and colleagues for an experiment with CHOK1 cells (compare Fig. 2C with Fig. 6B in Ref. 52). The reproducibility of the results obtained with the two different methods of enrichment for nascent DNA in the endogenous ori- $\beta$  locus in CHOK1 cells argues strongly that the application of our methods to analysis of the ectopic ori- $\beta$  fragments in DR12 hamster cells accurately reflects the initiation activity emanating from the ectopic fragments.

The initiation activity of exogenous ori- $\beta$  in uncloned pools of stably transfected cells was at least as great as that observed for endogenous ori- $\beta$  in CHOK1 cells (compare Fig. 2C and Fig. 3C). This result indicates that ori- $\beta$  does not require either ori- $\beta'$  or ori- $\gamma$  to direct initiation of chromosomal DNA replication. Since the ectopic ori- $\beta$  fragments were incorporated at random in the genome of the transfected cells, the exogenous DNA

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fragments could conceivably integrate into a chromosomal context that directed efficient initiation from the ori- $\beta$  IR. However, this possibility seems unlikely since at least some initiation activity was detected at the ectopic ori- $\beta$  IR in each individual cell clone tested (Table 3). A simpler interpretation is that the 5.7 kb fragment contains all of the sequences necessary to specify the start sites for replication at ori- $\beta$ , and hence serves as an independent chromosomal replicator. Further support for this interpretation is provided by the demonstration that specific DNA sequences within the 5.7 kb fragment were necessary for efficient initiation at the ori- $\beta$  start site (Fig. 4, Table 4).

Although the exogenous 5.7 kb ori- $\beta$  fragment was functional to some degree in at least six random ectopic locations, the initiation activity of the ori- $\beta$  fragment varied among the individual cell clones from just above background to about half of that detected in uncloned pools of transfectants (Table 3). This clonal variation may reflect position effects exerted by the flanking chromatin that were masked when ori- $\beta$  activity was measured in uncloned pools of pMCD-transfected cells (Fig. 3C). The notion that ectopic ori- $\beta$  was subject to position effects would be consistent with the lower initiation activity of the ectopic fragments in the cloned lines compared to that in uncloned pools of transfected cells. It should be noted that initiation activity of the ectopic ori- $\beta$  fragment in the pools of transfectants was determined four to five weeks after transfection, whereas the initiation activity of the ori- $\beta$  fragment in the subclones was determined fourteen weeks after transfection due to the time required to expand the cloned cells. The decreased initiation activity of the subclones could thus be due to progressive chromatin-mediated repression of the integrated ori- $\beta$  fragment over time. Gradual extinction of gene expression from stably transfected genes has been frequently observed after long-term

propagation of transfected cells, but was avoided when the transfected genes were flanked by insulators (73). Similarly, *Drosophila* amplification control elements placed at ectopic sites have been shown to be subject to position effects that were prevented by flanking the ACE element with insulators (60).

The possibility that ectopic ori- $\beta$  may be subject to position effects raises the question of how the endogenous DHFR initiation zone escapes position effects and whether it may be protected by elements such as insulators. Interestingly, a 3.2 kb fragment at the 3' end of the DHFR coding sequence in the endogenous locus appeared to be required for all replication activity in the 55 kb initiation zone (49). This 3.2 kb DNA fragment was not included in the 5.7 kb ori- $\beta$  fragment shown here to function as an independent replicator at ectopic sites in uncloned pools of stably transfected DR12 cells (Fig. 3). However, given that the 3.2 kb sequence in the endogenous DHFR locus flanks the initiation zone at one end, one possibility is that it may promote replication over the entire zone by insulating it from position effects that would prevent its initiation activity. Although other possible functions for this element can also be imagined, this speculation makes specific predictions that could be tested experimentally.

In the endogenous DHFR locus, the three preferred replication start sites in the initiation zone have been suggested to represent redundant genetic elements (72). One prediction of this hypothesis is that each preferred site should be sufficient, on its own, to direct initiation of DNA replication. Our data show that this prediction is met by ori- $\beta$ . Another prediction is that deletion of one of the preferred start sites from the endogenous locus might be compensated by increased activity at the remaining two preferred start sites. Consistent with this prediction, deletion of a 4.5 kb ori- $\beta$  sequence

in the endogenous locus, overlapping the 5.7 kb ori- $\beta$  fragment used in our study, failed to reduce initiation activity in a broad zone that retained ori- $\beta'$  and ori- $\gamma$ , as detected by two-dimensional gel electrophoresis (49). Since this 4.5 kb deletion encompassed two of the same regions in the 5.7 kb fragment that we found to be critical for initiation activity (pMCD $\Delta$ AT and pAKO in Fig. 4), we speculate that ori- $\beta'$  and ori- $\gamma$  may be sufficient to direct initiation over the entire zone in the endogenous locus. It would be interesting to determine whether either the ori- $\beta'$  or ori- $\gamma$  region could also serve as an independent chromosomal replicator in an ectopic location. The working model that the preferred start sites in the endogenous DHFR locus are redundant may provide additional insight into the complex patterns of replication intermediates observed for the 55 kb initiation zone by 2D gel electrophoresis (86, 41, 28, 26, 89).

**Is ori-  $\beta$  composed of essential modular elements?** Studies of model systems have shown that replicators usually have a modular organization (reviewed in 22). The origin core consists of discrete elements: a DNA unwinding element (DUE), an origin recognition element (ORE) that contains binding sites for initiator proteins, and sometimes an AT-rich region. The core is often flanked by auxiliary elements that bind transcription factors and enhance the replication activity of the core element up to 1000-fold.

In the ectopic DHFR ori- $\beta$ , at least two well-separated DNA sequence elements were critical for full activity (Fig. 4, Table 4). Deletion of the GA dinucleotide repeat in pMCD $\Delta$ DNR or the central AT-rich sequence in pMCD $\Delta$ AT reduced initiation activity nearly 10-fold. The markedly different sequence composition of these two elements

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suggests that they probably serve different functions. Although these functions remain to be elucidated, several possible functions are suggested by previous studies. For example, GA dinucleotide repeats direct the establishment of a functionally important nucleosomal array in the transcription control region upstream of a *Drosophila* heat shock (61) and could play such a role in the ectopic DHFR ori- $\beta$ . The central AT-rich region that was deleted in pMCD $\Delta$ AT harbors a potential DUE (16) and sequences homologous to a cell cycle-dependent protein footprint in the human lamin B2 IR (39, 29, 1, 25) and to the ORC binding sites in ACE3 (6, 80). Either DNA unwinding or protein binding could account for the requirement for the central AT-rich region in the 5.7 kb fragment of ori- $\beta$ .

A deletion of only four base pairs in the 5.7 kb ori- $\beta$  fragment (pAKO) cut its initiation activity by half (Fig. 4D). Although this result was initially quite surprising, inspection of the DNA sequences around the deletion suggests at least two possible explanations for the striking effect of this mutation. The mutation removed four GC bp from a six bp run of GC sequence, one of only two such GC hexanucleotides in an AT-rich region of 1.2 kb between the pMCD $\Delta$ AT and pMCD $\Delta$ DNR mutations (Fig. 4A). Maintenance of this stretch of GC may be important for full initiation activity. Another possibility is that the 4 bp deletion altered the spacing between flanking modular elements that must cooperate to initiate replication. For example, insertions of approximately half of a DNA helical turn between neighboring elements in the SV40 early promoter was reported to decrease transcription activity by about 90%, whereas separation of the elements by a full helical turn was consistently less detrimental (81). Interestingly, the 4 bp deletion in pAKO resides just three nucleotides downstream of an AT-rich element that is homologous to ORC binding sites in ACE3 (Fig. 4A) and may bind to hamster

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ORC protein (cited in 12). If ORC binding in this region indeed plays a role in ori- $\beta$  activity, the 4 bp deletion may disrupt ORC interactions with flanking elements such as the stably bent region and the RIP60 binding sites whose functional importance remains unknown (15, 16).

In contrast with the other mutations, deletion of the upstream T-rich element (pMCD $\Delta$ TR) more than tripled the activity of the DHFR ori- $\beta$  IR (Fig. 4D), indicating that the T-rich element is not required for activity of the 5.7 kb fragment in ectopic sites. The deleted sequences encompassed an Alu repeat, a previously described cell cycle-dependent nuclease hypersensitive site, and sequences homologous to ORC binding sites in ACE3 and to *S. pombe* ARS elements, (Fig. 4A), indicating that none of these elements is essential for ori- $\beta$  activity. One possible interpretation of these data is that the T-rich element may limit the initiation activity of ori- $\beta$ , either only in the ectopic fragment, or perhaps also in the endogenous locus. Deletion of the T-rich element might eliminate a protein binding site that competes with the proposed ORC binding site (12) immediately downstream from the deletion (Fig. 4A, checkered boxes). Alternatively, the deletion may affect chromatin structure, increasing protein access to the origin or promoting unwinding at the ori- $\beta$  IR.

In summary, the results presented here strongly suggest that DNA sequences in the 5.7 kb DHFR fragment are sufficient to direct efficient initiation at the ori- $\beta$  IR in multiple ectopic chromosomal sites and that initiation activity depends on discrete genetic elements located at or near the IR. Further characterization of these elements will be required to confirm their proposed modular nature and to elucidate their biochemical functions.

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## FIGURE LEGENDS

**Fig.1. (A)** Features of the endogenous DHFR ori- $\beta$  initiation region (IR). Preferred start sites of DNA replication, ori- $\beta$ , ori- $\beta'$ , and ori- $\gamma$ , a 55 kb initiation zone (shaded area) between the DHFR and 2BE2121 coding sequences, and matrix attached regions (MARs, stippled boxes) are indicated (43, 44, 5, 42, 55, 14, 85, 86, 71, 52, 89, 57). The 5.7 kb fragment of the DHFR ori- $\beta$  (pMCD), extending from the Bam HI to the Kpn I recognition sequences, is indicated. Arrows denote the position of the primers used in the competitive PCR assay for origin function and correspond to primer pairs used in previous DHFR ori- $\beta$  mapping studies (71).

**(B)** Strategy for quantitating initiation occurring in exogenous DHFR ori- $\beta$  fragments in ectopic chromosomal locations. A 5.7 kb wildtype or mutated DHFR ori- $\beta$  fragment was co-electroporated with a neomycin resistance gene into the DR12 cell line, a CHO derivative containing a 150 kb deletion encompassing the entire DHFR locus. After selection with G418, total DNA was isolated, heat-denatured and size-fractionated on a 5-30% linear neutral sucrose gradient. The fraction containing single-stranded DNA with a length of 1-2 kb, representing nascent DNA, was isolated and the abundance of ori- $\beta$  target sequences contained in the fraction was quantitated by competitive PCR.

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**Fig. 2.** Initiation of DNA replication at the endogenous DHFR ori- $\beta$  in asynchronous CHOK1 cells. (A) PCR amplification reactions were performed with each of the four primer pairs and size-fractionated nascent DNA template from asynchronous CHOK1 cells in the presence of a pre-calibrated amount of the corresponding competitor DNA. Amplification products were analyzed by polyacrylamide gel electrophoresis and ethidium bromide staining. Control lanes: C, competitor template only; G, nascent genomic DNA template only; -, no template. Numbers above each lane represent the volume ( $\mu$ l) of nascent DNA added to the PCR reaction. Amplification reactions with pp2 used a 1:10 dilution of the nascent DNA.

(B) PCR reactions were performed and analyzed as in panel A, except that the template was a 1:10 dilution of sheared, denatured total genomic CHOK1 DNA of 1-2 kb in length.

(C) Amplification products generated with either nascent DNA from asynchronous CHOK1 cells (black bars) or sheared total genomic DNA from asynchronous CHOK1 cells (white bars) were quantitatively evaluated for five independent experiments with each type of template. As a measure of initiation activity, the abundance of each target sequence in nascent genomic DNA was normalized to the abundance of pp3 target sequences in the corresponding experiment, which was set equal to 1 (see example in Table 2), and the average of five experiments is shown. Bars indicate the SEM.

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**Fig. 3.** Initiation of DNA replication in the exogenous 5.7 kb ori- $\beta$  fragment in DR12

cells. (A) The integrated exogenous DHFR ori- $\beta$  fragment in 5 ng of DNA from uncloned pools of DR12 cells and the endogenous ori- $\beta$  in 5 ng of CHOK1 DNA were amplified by PCR. The ratio of the DR12 amplification product relative to that of CHOK1 is indicated.

(B) PCR amplification reactions were performed with each of the four primer pairs and size-fractionated nascent DNA from asynchronous pMCD-transfected DR12 cells in the presence of a pre-calibrated amount of the corresponding competitor DNA. Amplification products were analyzed by polyacrylamide gel electrophoresis and ethidium bromide staining. Control lanes: C, competitor template only; G, nascent genomic DNA template only; -, no template. Numbers above each lane represent the volume ( $\mu$ l) of nascent DNA added to the PCR reaction. Amplification reactions for pp2 and pp6 used a 1:10 dilution of the nascent DNA.

(C) Amplification products generated with either nascent DNA from a pool of asynchronous pMCD-transfected DR12 cells (black bars) or sheared total genomic DNA from asynchronous pMCD-transfected DR12 cells (white bars) were quantitatively evaluated for seven independent transfection experiments. As a measure of initiation activity, the abundance of each target sequence in nascent genomic DNA was normalized to the abundance of pp3 target sequences in the corresponding experiment, which was set equal to 1 (see example in Table 2), and the average of the seven experiments is shown. Bars indicate the SEM.

**Fig. 4.** Initiation of DNA replication in wildtype and mutant exogenous ori- $\beta$  DNA fragments. (A) Unusual DNA sequences in the 5.7 kb ori- $\beta$  fragment and the location of the deletion mutants are indicated on the restriction map of the region. Positions of Alu I repetitive elements (white boxes) and AT-rich sequences homologous to the *S. pombe* origin consensus motifs D, C, Z and M (50) (small black boxes) are marked. AT-rich sequences homologous to a cell cycle-dependent DNase I genomic footprint in the human lamin B2 IR (39, 29, 1) (hatched box) and to the ORC-binding region in *Drosophila* chorion amplification control element ACE3 (6, 80) (checkered boxes) are noted. AT-rich sequences containing stably bent DNA (B) and binding sites for a zinc finger protein of unknown function RIP60 (black arrowheads) are indicated (21, 15, 16, 64, 45). The position of a cell-cycle dependent nuclease hypersensitive site (72) is indicated (striped box).

(B) The integrated mutant DHFR ori- $\beta$  fragments in 5 ng DNA from uncloned pools of DR12 cells and the endogenous ori- $\beta$  in 5 ng of CHOK1 DNA were amplified by PCR and visualized by gel electrophoresis and ethidium bromide staining. The ratio of the mutant amplification products relative to that of CHOK1 is indicated.

(C) PCR amplification reactions were performed with each of the four primer pairs and size-fractionated nascent DNA from asynchronous mutant-transfected DR12 cells in the presence of a pre-calibrated amount of the corresponding competitor DNA. Amplification products were analyzed by polyacrylamide gel electrophoresis and ethidium bromide staining. Control lanes: C, competitor template only; G, nascent genomic DNA template only; -, no template. Numbers above each lane represent the volume ( $\mu$ l) of nascent DNA

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added to the PCR reaction; note that for pMCD $\Delta$ TR, the nascent genomic DNA was used at a 1:10 dilution with pp2 and pp6.

(D) The abundance of nascent DNA from pools of mutant-transfected DR12 cells from three independent transfections experiments was quantitatively evaluated. As a measure of initiation activity, the abundance of each target sequence in nascent genomic DNA was normalized to the abundance of pp3 target sequences in the corresponding experiment, which was set equal to 1 (see Table 4 for a typical experiment), and the average of three experiments with each mutant is shown. For comparison, the average initiation activity measured with nascent DNA from a pool of asynchronous wild type pMCD-transfected DR12 cells (Fig. 3C) is also shown. Bars indicate the SEM.

Table 1: Oligonucleotide primer sequences and position relative to the Bam H1 site of pMCD

PRIMER	SEQUENCE 5' - 3'	position <sup>a</sup>
8SX	CTCTTCATAGTTCTCAGGC	470-489
8DX	GTCCTCGGTATTAGTTCTCC	651-670
2SX	GTCCCTGCCTCAAACACAA	1070-1089
2DX	CCTTCATGCTGACATTTGTC	1329-1348
6SX	AACTGGCTTCCCAAGAAATT	1517-1536
6DX	AACCTCTGAACTGTAAGCTG	1666-1685
3SX	GGACACTAAGTCTAGGTACTACA	3882-3904
3DX	GCTGGGATAAGTTGAAATCC	4121-4140
8SXCOM <sup>b</sup>	<u>GTCGACGGATCCCTGCAGGTCATTCATCAAGCTGGAAAGC</u>	529-548
8DXCOM	<u>ACCTGCAGGGATCCGTCGACTCCATGGCAGTCTTCACACT</u>	549-569
2SXCOM	<u>GTCGACGGATCCCTGCAGGTAAGGAAGGAAAGAAAGGGCCC</u>	1126-1146
2DXCOM	<u>ACCTGCAGGGATCCGTCGACCTCAGTGAGTCCACTTGCTTT</u>	1147-1168
6SXCOM	<u>GTCGACGGATCCCTGCAGGTATAGAAACCCAGCTAAGAC</u>	1587-1606
6DXCOM	<u>ACCTGCAGGGATCCGTCGACTGCTGTGAAGAGACACCATG</u>	1607-1626
3SXCOM	<u>GTCGACGGATCCCTGCAGGTTAGGAAACTGAGATGCCAGG</u>	3992-4010
3DXCOM	<u>ACCTGCAGGGATCCGTCGACAGGACTCAGCTCTTACTAAC</u>	4011-4031

<sup>a</sup>nucleotide position relative to the BamHI recognition sequence in the DHFR ori- $\beta$  fragment pMCD, defined as position 1 (Genbank Accession # Y09885).

<sup>b</sup>underlined sequence is the 5' tail of the PCR primer which gave rise to the 20 bp insertion in the competitor plasmids (see Materials and Methods for construction).

Table 2: Abundance of DHFR ori- $\beta$  target sequences in nascent DNA<sup>a</sup>

	pp8	pp2	pp6	pp3
Nascent CHOK1 abundance <sup>b</sup>	$8.49 \times 10^{-6}$	$2.80 \times 10^{-4}$	$3.65 \times 10^{-5}$	$2.18 \times 10^{-5}$
Initiation activity <sup>c</sup>	0.4	12.8	1.7	1.0
Nascent pMCD pool abundance	$1.32 \times 10^{-5}$	$2.59 \times 10^{-4}$	$6.63 \times 10^{-5}$	$1.02 \times 10^{-5}$
Initiation activity	1.3	25.4	6.5	1.0
Total sheared DNA abundance	$3.45 \times 10^{-5}$	$1.37 \times 10^{-4}$	$1.03 \times 10^{-4}$	$1.06 \times 10^{-4}$
Initiation activity	0.3	1.3	1.0	1.0

<sup>a</sup>Values in the table are from a typical experiment.

<sup>b</sup>Nascent abundance is calculated as described in Materials and Methods.

<sup>c</sup>Initiation activity is calculated as described in Materials and Methods.

Table 3: Abundance of target nascent DNA chains in the DHFR ori- $\beta$  initiation zone<sup>a</sup>

	Copy # <sup>d</sup>	pp8	pp2	pp6	pp3
Nascent clone 1 abundance <sup>b</sup>	1	$2.04 \times 10^{-5}$	$2.49 \times 10^{-4}$	$1.41 \times 10^{-5}$	$3.62 \times 10^{-5}$
Initiation activity <sup>c</sup>		0.6	6.9	0.4	1.0
Nascent clone 2 abundance	0.7	$6.16 \times 10^{-6}$	$5.03 \times 10^{-5}$	$7.73 \times 10^{-6}$	$6.79 \times 10^{-6}$
Initiation activity		0.9	7.4	1.1	1.0
Nascent clone 3 abundance	0.9	$8.11 \times 10^{-6}$	$7.05 \times 10^{-5}$	$7.09 \times 10^{-6}$	$2.04 \times 10^{-5}$
Initiation activity		0.4	3.5	0.4	1.0
Nascent clone 4 abundance	0.8	$4.07 \times 10^{-6}$	$6.04 \times 10^{-5}$	$5.71 \times 10^{-6}$	$1.05 \times 10^{-5}$
Initiation activity		0.4	5.8	0.5	1.0
Nascent clone 5 abundance	1	$3.03 \times 10^{-6}$	$6.87 \times 10^{-5}$	$8.50 \times 10^{-6}$	$7.31 \times 10^{-6}$
Initiation activity		0.4	9.4	1.2	1.0
Nascent clone 6 abundance	0.7	$8.77 \times 10^{-6}$	$3.88 \times 10^{-5}$	$7.36 \times 10^{-6}$	$1.45 \times 10^{-5}$
Initiation activity		0.6	2.7	0.51	1.0

<sup>a</sup>Values in the table are from a typical experiment with each clone.

<sup>b</sup>Nascent abundance is calculated as described in Materials and Methods.

<sup>c</sup>Initiation activity is calculated as described in Materials and Methods.

<sup>d</sup>Integration ratio of ori- $\beta$  fragment relative to endogenous ori- $\beta$  in CHOK1 cells.

Table 4: Abundance of DHFR ori- $\beta$  target sequences in nascent DNA from deletion mutants<sup>a</sup>

	pp8	pp2	pp6	pp3
Nascent pMCD pool abundance <sup>b</sup>	$1.13 \times 10^{-4}$	$9.10 \times 10^{-4}$	$2.00 \times 10^{-4}$	$3.08 \times 10^{-5}$
Initiation activity <sup>c</sup>	3.7	29.6	6.5	1.0
Nascent pMCD $\Delta$ DNR pool abundance	$4.18 \times 10^{-6}$	$3.56 \times 10^{-5}$	$2.69 \times 10^{-5}$	$1.44 \times 10^{-5}$
Initiation activity	0.3	2.5	1.9	1.0
Nascent pMCD $\Delta$ AT pool abundance	$1.98 \times 10^{-5}$	$1.18 \times 10^{-4}$	$4.45 \times 10^{-5}$	$2.73 \times 10^{-5}$
Initiation activity	0.7	4.3	1.6	1.0
Nascent pAKO pool abundance	$1.54 \times 10^{-5}$	$3.34 \times 10^{-4}$	$6.56 \times 10^{-5}$	$4.52 \times 10^{-5}$
Initiation activity	0.4	7.6	1.5	1.0
Nascent pMCD $\Delta$ TR pool abundance	$1.26 \times 10^{-7}$	$1.20 \times 10^{-5}$	$4.66 \times 10^{-6}$	$1.01 \times 10^{-7}$
Initiation activity	1.3	118.0	45.9	1.0
Total DNA abundance (pMCD pool)	$1.60 \times 10^{-6}$	$5.60 \times 10^{-6}$	$3.37 \times 10^{-6}$	$4.18 \times 10^{-6}$
Initiation activity	0.4	1.3	0.8	1.0

<sup>a</sup>Values in the table are from a typical experiment.

<sup>b</sup>Nascent abundance is calculated as described in Materials and Methods.

<sup>c</sup>Initiation activity is calculated as described in Materials and Methods.

FIGURE 1

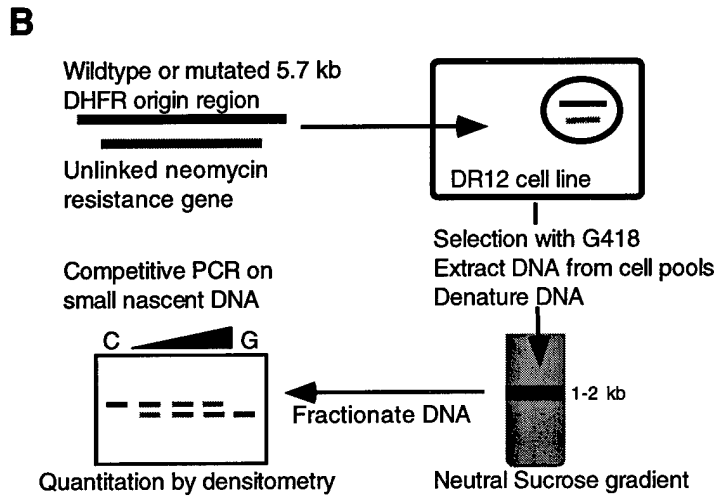
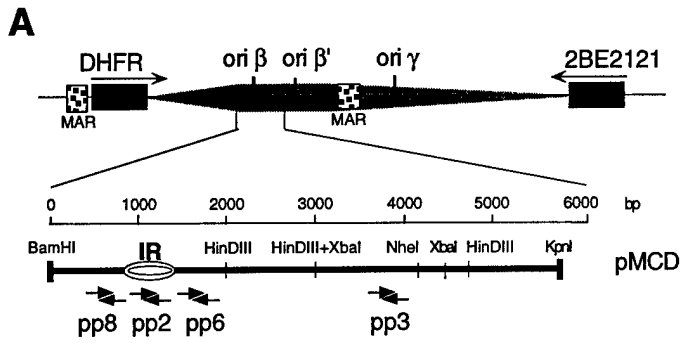


FIGURE 2

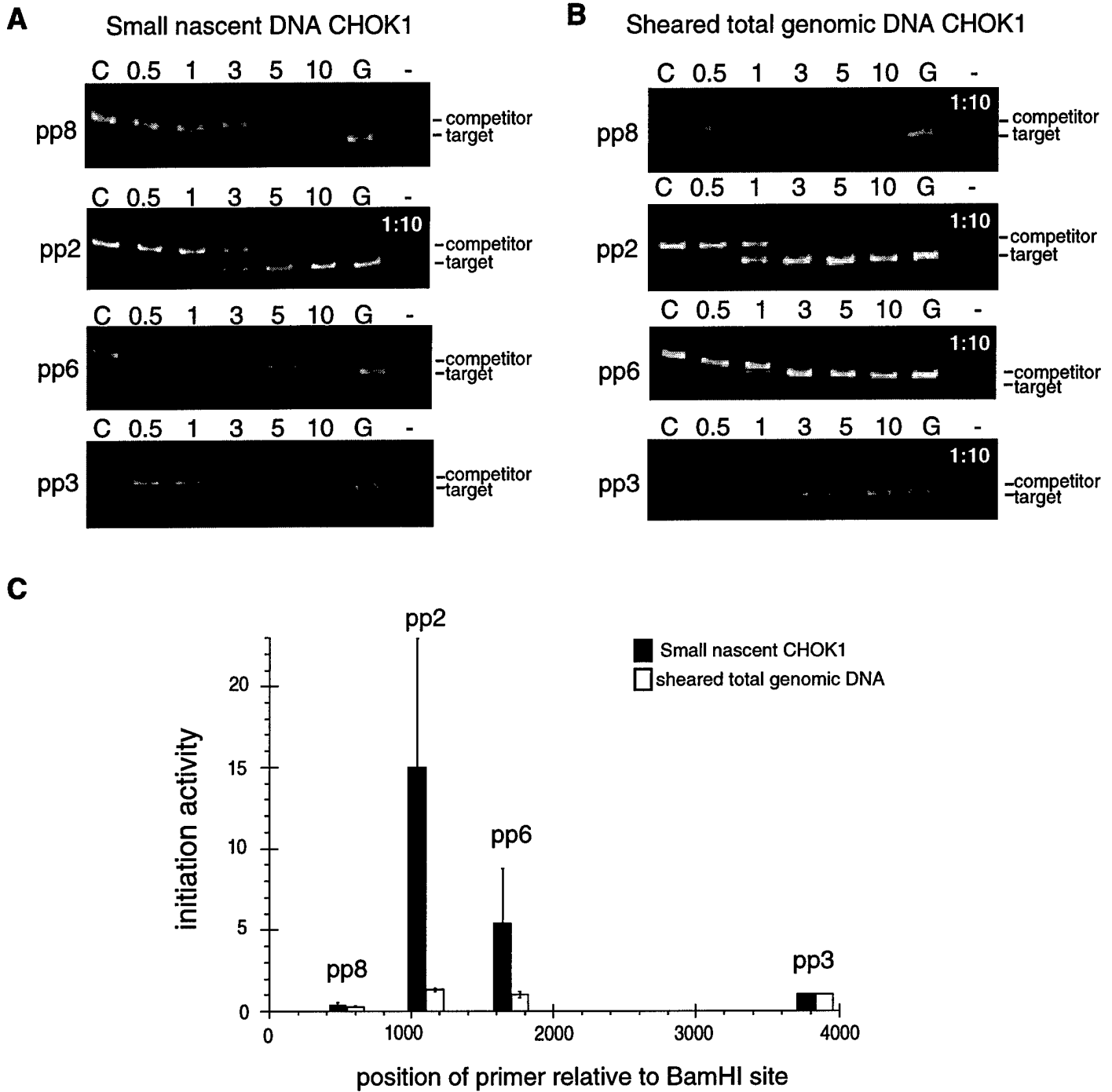


FIGURE 3

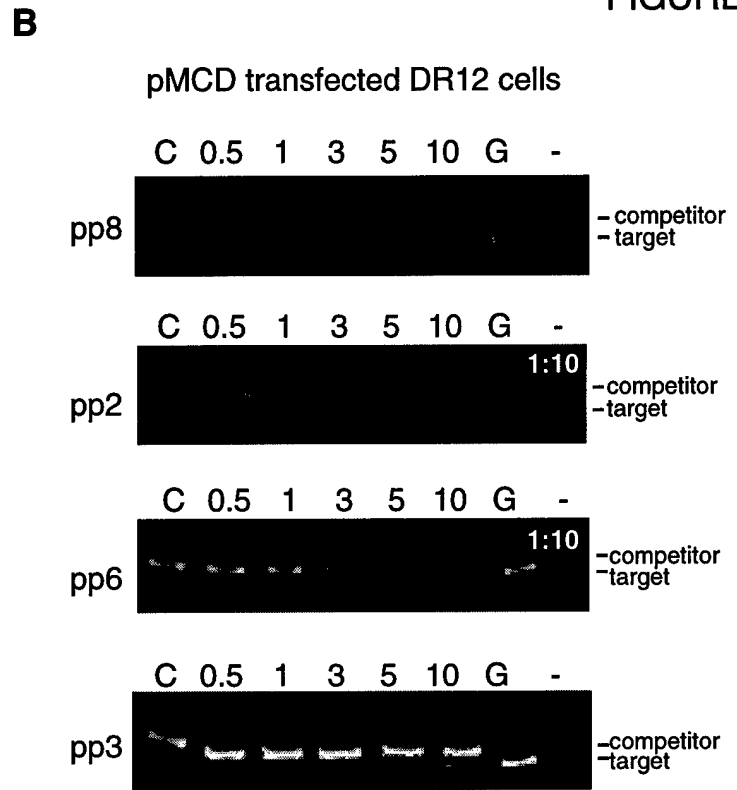
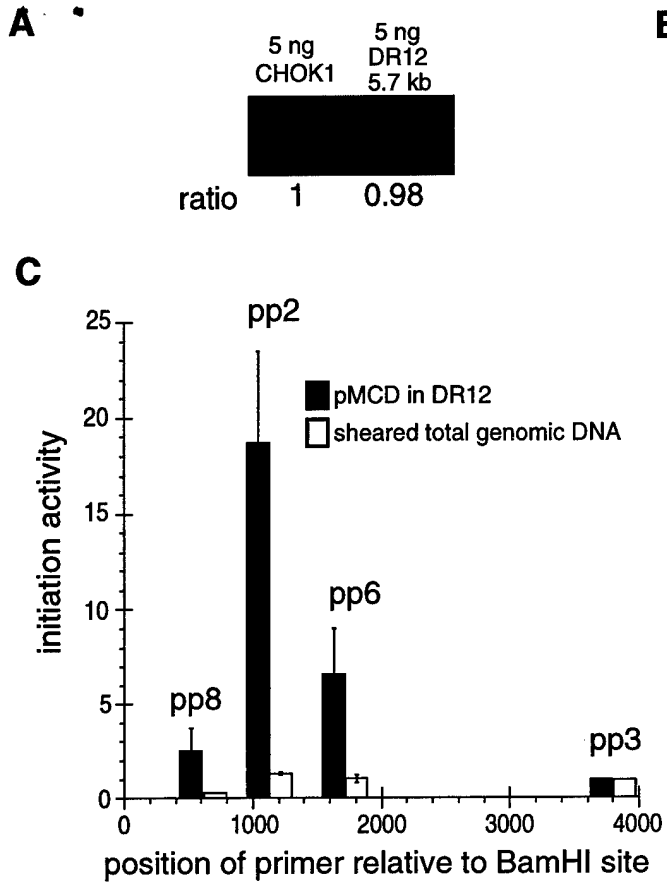


FIGURE 4

