

Mapping an Origin of DNA Replication at a Single-Copy Locus in Exponentially Proliferating Mammalian Cells

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A general method for determining the physical location of an origin of bidirectional DNA replication has been developed recently and shown to be capable of correctly identifying the simian virus 40 origin of replication (L. Vassilev and E. M. Johnson, *Nucleic Acids Res.* 17:7693–7705, 1989). The advantage of this method over others previously reported is that it avoids the use of metabolic inhibitors, the requirement for cell synchronization, and the need for multiple copies of the origin sequence. Application of this method to exponentially growing Chinese hamster ovary cells containing the nonamplified, single-copy dihydrofolate reductase gene locus revealed that DNA replication begins bidirectionally in an initiation zone approximately 2.5 kilobases long centered about 17 kilobases downstream of the DHFR gene, coinciding with previously described early replicating sequences. These results demonstrate the utility of this mapping protocol for identifying cellular origins of replication and suggest that the same cellular origin is used in both the normal and the amplified DHFR locus.

Identification of origins of DNA replication in mammalian chromosomes has remained an elusive goal (13). Several mammalian sequences have been identified which promote autonomous replication of plasmids in mammalian cells, but, so far, only one has been associated with a chromosomal origin of replication (11). Methods for identifying mammalian origins of replication that function in the chromosome have focused on regions amplified up to 1,000 times in cells synchronized at their G₁/S boundary by nutritional deprivation and DNA synthesis inhibition (1–3, 8, 9). So far, two methods have been used to locate mammalian origins at a single-copy locus (7, 11). However, the resolution of these methods is limited, and one of them (7) requires extensive inhibition of protein synthesis.

In an effort to overcome these problems, a general method was developed for determining the physical location of an origin of bidirectional DNA replication, and its validity was demonstrated by using the simian virus 40 genome (14). This method determines the distance from specific DNA sequence markers to a nearby origin of replication by labeling nascent DNA chains *in vivo*, fractionating them according to size, and then identifying the shortest nascent DNA chains that have traversed specific DNA sequence markers on either side of the origin by blotting and hybridization with ³²P probes directed against the specific sequence markers (see Fig. 1). Since single-copy sequences in replication intermediates from mammalian cells are virtually undetectable by hybridization, they are first amplified by using the polymerase chain reaction (PCR). The advantages of this method are that it avoids the use of metabolic inhibitors, it does not require synchronized cells, and it can detect replication through single-copy sequences.

The utility of this approach for mapping origins of replication in mammalian chromosomes has now been evaluated. Analysis of exponentially growing Chinese hamster ovary (CHO) cells containing a single copy of the dihydrofolate reductase (DHFR) gene locus per haploid genome revealed

an origin of bidirectional DNA replication located in a region of about 2.5 kilobases (kb) centered approximately 17 kb downstream of the DHFR gene. This "initiation zone" coincides with a region for initiation of DNA replication previously identified in CHO cells containing a 500-fold amplification of the DHFR locus, suggesting that the same cellular origin is used in both the normal and amplified sequence. In separate experiments, an initiation zone was also identified upstream of the 5' end of the human *c-myc* gene (14a). These results demonstrate the applicability of this mapping protocol to identifying cellular origins of replication in mammalian cells.

MATERIALS AND METHODS

Labeling and isolation of nascent cellular DNA chains. Chinese hamster ovary cells (CHO-K1) were grown in 150-cm² flasks with Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (GIBCO Laboratories) and nonessential amino acids. For each experiment, 12 flasks containing cells that were about 50% confluent were radiolabeled with 3 μ Ci of ³H-deoxycytidine ([³H]dC, 19 Ci/mmol; Amersham Corp.) per ml and 20 μ M 5-bromodeoxyuridine (BrdU; Boehringer Mannheim Biochemicals) for 15 min at 37°C. Since BrdU-labeled DNA is sensitive to UV-induced strand breaks, all of the following procedures should be done under minimal light. Cells in each flask were washed three times with cold phosphate-buffered saline and lysed by the addition of 7 ml of 50 mM Tris hydrochloride (pH 8.0)–1 M NaCl–10 mM EDTA–0.5% sodium dodecyl sulfate. Lysates were combined and incubated overnight with 100 μ g of proteinase K (Boehringer) per ml. DNA was isolated by gentle inversions in phenol-chloroform (1:1) to avoid shearing, followed by spooling on glass rods under 70% ethanol. The spooled DNA was rinsed in 95% ethanol and then dissolved in 4 ml of 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA (TE buffer) by reversing the direction of spooling. NaOH (1 N) was then added to give a final concentration of 0.2 N NaOH, and the DNA was fractionated by sedimentation through 5 to 20% (wt/vol) linear

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sucrose gradients containing 0.2 N NaOH and 2 mM EDTA for 18 h at 15°C in a Beckman SW28 rotor at 24,000 rpm.

Gradients were fractionated from the top to avoid contamination by viscous high-molecular-weight DNA at the bottom of the tube. Corresponding fractions from two identical gradients were combined. Fractions 12 to 14 were discarded because resolution in this region of the gradient was poor. Fraction 1 was discarded because a significant portion of its DNA was shorter than the PCR amplification segments. Fractions 2 to 11 were renumbered 1 to 10 for convenience. Each fraction was adjusted to 0.1 M Tris hydrochloride (pH 7.5) before neutralizing with 2 N HCl. The amount of ^3H label (determined on 50- μl samples) depended upon the fraction of actively proliferating cells but was generally ~ 600 cpm/ μg of DNA. Each gradient fraction was supplemented with 100 μg of carrier DNA (salmon sperm DNA that had been degraded by boiling in 1 N NaOH for 1 h and neutralized) before cellular DNA was ethanol precipitated.

DNA from each fraction was dissolved in 0.5 ml of TE buffer, heat denatured at 95°C for 3 min in a 1.5-ml Eppendorf microcentrifuge tube, rapidly cooled to room temperature by using an ice bath, and adjusted to 10 mM sodium phosphate (pH 7.0), 0.14 M NaCl, and 0.05% Triton X-100 (immunoprecipitation buffer). Each fraction was incubated with 80 μl of mouse anti-BrdU monoclonal antibody (25 $\mu\text{g}/\text{ml}$, Becton Dickinson, San Jose, Calif.) for 20 min at room temperature with constant shaking. Rabbit immunoglobulin G directed against mouse immunoglobulin G (15 μl of 2.3 mg/ml; Sigma Chemical Co.) was then added to precipitate BrdU-DNA-antibody complexes (14, 15). Immunoprecipitates were collected by centrifugation for 5 min, washed once with 0.5 ml of immunoprecipitation buffer, and then resuspended in 200 μl of 50 mM Tris hydrochloride (pH 8.0)–10 mM EDTA–0.5% sodium dodecyl sulfate–250 μg of proteinase K per ml. In this procedure, care must be taken not to lose the precipitate. DNA pellets were thoroughly homogenized with a vortex device, digested overnight at 37°C, combined with 100 μg of carrier DNA (prepared as described above), extracted once with an equal volume of phenol-chloroform (1:1), adjusted to 0.3 M sodium acetate, and then precipitated in ethanol as before. DNA was redissolved in 100 μl of TE buffer, subjected to a second round of immunoprecipitation, and then precipitated with ethanol in the presence of 20 μg of *Escherichia coli* tRNA (Sigma) instead of carrier DNA. These fractions were dissolved in 50 μl of TE buffer and used for PCR amplification.

PCR conditions. Three segments referred to as A, B, and C were chosen from the sequence of 6,157 base pairs bounded by *Xba*I and *Hind*III DNA restriction sites and located downstream of the DHFR gene (4; see Fig. 5). For each segment, two PCR primers 20 residues in length and one hybridization probe 21 residues in length were chemically synthesized as described previously (14). Nucleotide positions of each primer pair from the *Xba*I site were as follows: segment A, 5'-81 to 100, 3'-381 to 400; segment B, 5'-2677 to 2696, 3'-2976 to 2995; and segment C, 5'-4569 to 4588, 3'-4955 to 4974. Nucleotide positions of each probe were as follows: segment A, 5'-145 to 165; segment B, 5'-2787 to 2807; and segment C, 5'-4643 to 4662. PCR amplification reactions were carried out in parallel with 5- μl samples of each purified nascent DNA fraction and the products were purified as previously described (14), except that the annealing reaction was carried out at 52°C for 2 min and the extension reaction was carried out at 72°C for 1 min with 2.5 U of Taq polymerase (Perkin-Elmer-Cetus) and 30 cycles of amplification in a Perkin-Elmer-Cetus thermal cycler.

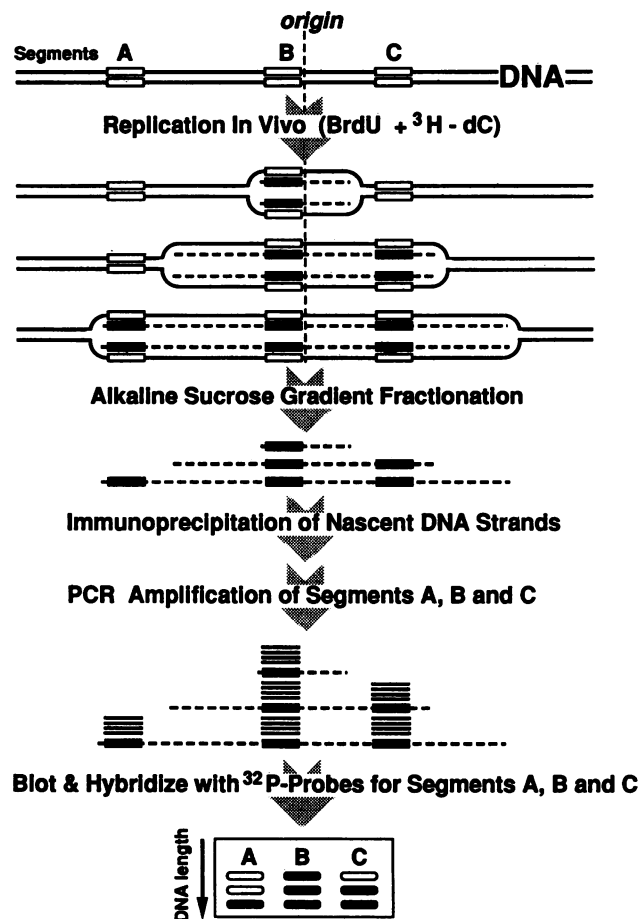


FIG. 1. Mapping an origin of bidirectional DNA replication in cellular chromosomes by measuring the lengths of nascent DNA chains.

Blotting and hybridization. Samples of the PCR products from each reaction were blotted to GeneScreen Plus membranes (Dupont, NEN Research Products) with a slot-blot device (Schleicher & Schuell) and hybridized with ^{32}P -labeled oligonucleotide probes ($\sim 3 \times 10^8$ cpm/ μg), as described previously (14). Following autoradiography, membranes were sliced into fractions and radioactivity was determined by liquid scintillation counting.

RESULTS

Identification of an initiation zone in the DHFR gene locus. The protocol outlined in Fig. 1 was applied to exponentially growing CHO cells containing a single copy of the DHFR gene locus per genome. Previous studies (1–3, 8, 9) suggested that DNA replication occurred at a specific site within this locus, producing a population of newly synthesized DNA chains with lengths that vary from the sizes of Okazaki fragments up to the size of mature replicons. Therefore, nascent DNA chains from exponentially proliferating, unsynchronized CHO cells were labeled with ^3H dC and BrdU and then sedimented through alkaline sucrose gradients to fractionate them according to size. The amount of radioactivity in each DNA chain corresponded closely with its average length, and the ratio of ^3H DNA to DNA length was nearly constant throughout the gradient, consistent with uniform labeling of nascent DNA chains.

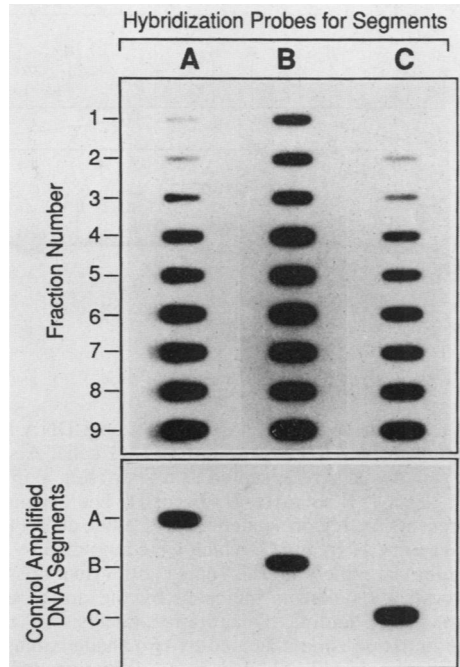


FIG. 2. Blotting and hybridization with ^{32}P probes for segments A, B, and C. (Upper panel) Nascent DNA in sucrose gradient fractions 1 through 9 (see Materials and Methods) was purified by immunoprecipitation, amplified by PCR, blotted to GeneScreen Plus, and independently hybridized with ^{32}P -oligonucleotide probes specific for segments A, B, and C (Fig. 1). (Lower panel) DNA segment A, B, or C in 1 μg of total CHO DNA was amplified with the same PCR primers as was the nascent DNA. Samples of the PCR products were then blotted and hybridized with the same ^{32}P -oligonucleotide probes specific for segments A, B, and C.

Since the partially purified nascent [^3H]DNA (20-fold increase in specific radioactivity) was contaminated with unlabeled parental DNA, nascent DNA in each sucrose gradient fraction was subjected to two rounds of immunoprecipitation with anti-BrdU antibodies (14, 15). An excess of carrier DNA was first added to each fraction to eliminate nonspecific precipitation of parental DNA. Under these conditions, 70 to 90% of the [^3H]BrdU-labeled CHO DNA in each fraction was precipitated. Immunoprecipitation of BrdU-labeled CHO DNA in the presence of pBR322 plasmid [^{32}P]DNA fragments demonstrated that less than 0.5% of non-BrdU-labeled DNA was retained in the first immunoprecipitation and less than 0.0004% was retained in the second immunoprecipitation.

Purified nascent DNA chains were used as templates to amplify the unique sequences in segments A, B, and C by using the PCR method. These three PCR-selected segments span about 5 kb overlapping the putative initiation zone identified in CHO 400 cells containing 1,000 copies of this region (see Fig. 5). Oligonucleotide primers for PCR were first tested in control reactions with total CHO DNA as a template. After 30 cycles of amplification, PCR products were phenol-chloroform extracted and fractionated by agarose gel electrophoresis. Each PCR primer pair generated a distinct DNA band corresponding in size to the length of the selected PCR segment, and the amount of PCR product formed was in proportion to the initial template concentration. Proportionality was maintained up to 0.1 μg of total DNA per 100- μl reaction. The standard error of the mean for seven identical samples that were PCR amplified simulta-

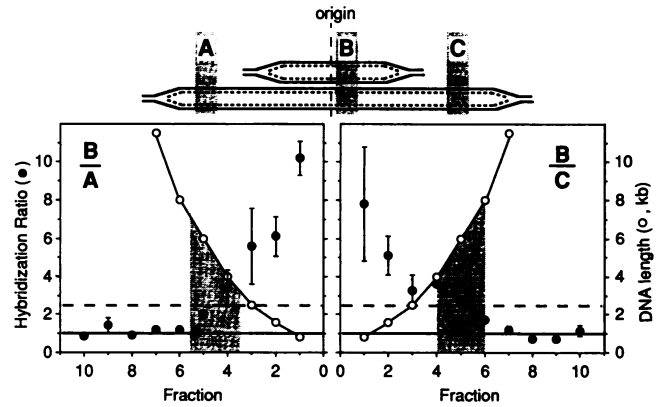


FIG. 3. Mapping an origin of DNA replication in the DHFR locus. Following autoradiography, each slot was excised and its radioactivity was measured by liquid scintillation counting. To correct for differences in hybridization efficiency, the amount of hybridization observed in each fraction was normalized to the average level observed in fractions 8 to 10. These fractions represented nascent DNA chains containing all three segments and therefore had B/A and B/C ratios of 1. For the remaining fractions, the B/A and B/C ratios were calculated from their normalized values and plotted as a function of fraction number and average DNA length determined from the data in Fig. 4A. Each point represents the average of two independent experiments (bars indicate ranges).

neously was 13% of the average value observed. Hybridization probes directed against segments A, B, and C detected strongly and specifically only the homologous PCR products. Samples of the purified nascent DNA fractions containing 5 to 75 ng of DNA were mixed with the PCR primers for segment A, B, or C and then amplified simultaneously in three parallel reactions. The PCR products were then blotted to a membrane support and hybridized with probes specific for segment A, B, or C (Fig. 2, upper panel). Samples of total CHO DNA were amplified in parallel with the nascent DNA fractions to confirm the specificity of hybridization for the probe (Fig. 2, lower panel).

The shortest nascent DNA chains contain only the segment closest to the origin, whereas longer chains will contain two or more segments (Fig. 1). Inspection of the hybridization pattern revealed which sucrose gradient fractions contained nascent DNA that was the minimum length necessary to include a given segment. Segments A and C were under-represented relative to segment B in fractions 1 through 4 or 5. Therefore, fragments of nascent DNA long enough to contain segments A and C were found predominantly in fractions 6 through 9. Only segment B hybridized to the smaller nascent DNA strands contained in fractions 1 through 5, indicating that segment B was closest to the origin of replication (see Fig. 5).

Mapping the chromosomal location of an origin of replication. Measuring the minimum length of nascent DNA necessary to include a given segment allows one to determine the distance from that segment to the origin of replication. Therefore, in order to determine the position of the origin between segments A and C, hybridization signals were quantified and the resulting data were used to calculate the ratios of ^{32}P -labeled probe that hybridized to B relative to A, and B relative to C, as a function of DNA length (Fig. 3). These ratios corrected for variations that may occur between different DNA segments during the amplification and hybridization steps.

Theoretically, if the nascent DNA chains in each fraction

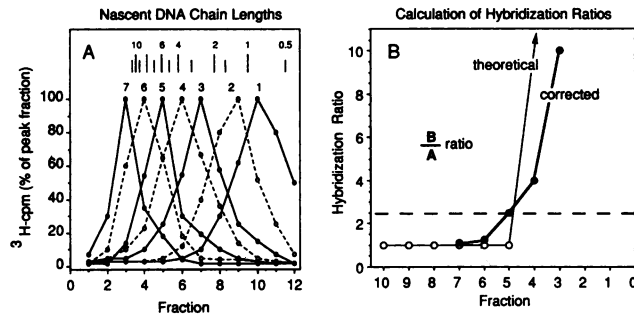


FIG. 4. (A) Lengths of nascent DNA chains in each sucrose gradient fraction determined by electrophoresis of samples in single 0.8% alkaline agarose gel (10). A "1 kilobase DNA ladder" (Bethesda Research Laboratories) was denatured and run in parallel to calibrate the gel (kilobase scale at top). The gel was neutralized, stained with ethidium bromide, and sliced into 12 fractions, and each fraction was melted in 0.5 ml of 0.1 N HCl with a microwave oven. ^3H -DNA was measured after the addition of 5 ml of Aquasol (Dupont, NEN) by liquid scintillation counting. The results from each lane were superimposed and presented as one figure (sucrose gradient fractions 1 to 7). (B) Theoretical and corrected ratios of ^{32}P probe hybridized to two different DNA segments. The percentage of DNA chains in each sucrose gradient fraction that were longer than 6 kb (the average peak value for fraction 5) were calculated from the peak areas of sucrose gradient fractions 3 through 7 (panel A). These numbers were 10% (fraction 3), 25% (fraction 4), 40% (fraction 5), 75% (fraction 6), and 90% (fraction 7). Using the formula $B/A = 100/x$, where x is the percentage determined above, the corrected B/A values were calculated and plotted together with the theoretical values for fractions 3 through 7 (see text).

of the sucrose gradient were identical in length, then the ratio of B/A or B/C would change from infinity to 1 as one passed from a fraction containing chains shorter than the minimum length to a fraction containing chains longer than the minimum length (Fig. 4B). However, each fraction actually contained a distribution of nascent DNA chain lengths that caused the observed ratio to change more gradually, depending on the extent of this size variation (Fig. 4A). Therefore, the population of chains within each fraction that were equal to or greater than the average chain length was calculated and used to determine the theoretically expected ratio of B/A or B/C that occurs at the shortest nascent DNA fragment (Fig. 4B). Since the B/A or B/C ratios will be 1 in those fractions with nascent DNA chains long enough to contain both A and B or C and B, then the corrected ratios will be equal to 100% divided by the percentage of DNA chains longer than the minimum length necessary to contain both A and B. For example, if the shortest nascent DNA chains containing segment A are in fraction 5 (average length, 6 kb), the theoretical curve for the B/A ratio will have a sharp transition between fractions 4 and 5 (Fig. 4B). However, the corrected curve in this case will define the shortest DNA chains that include segment A at a B/A ratio of 2.5 (broken line, Fig. 4B) instead of the theoretical ratio of 1. Therefore, the point at which the hybridization ratios change from less than 2.5 to greater than 2.5 marks the fraction containing the minimum nascent DNA chain length with both PCR segments present.

Thus, for the experimental data in Fig. 3, the shortest nascent DNA chains containing segment A + B or C + B will be found at B/A or B/C ratios, respectively, between 2 and 3 (broken line, Fig. 3) instead of 1 (solid line, Fig. 3). This occurred between fractions 4 and 5 for the B/A ratio and in fraction 5 for the B/C ratio. The maximum error in this

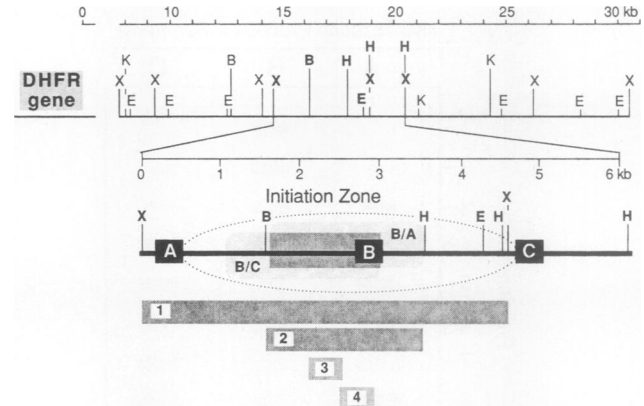


FIG. 5. Location of an origin of bidirectional DNA replication downstream from the DHFR gene in CHO-K1 cells. A segment of the DHFR gene locus is represented as a restriction map (X, *Xba*I; K, *Kpn*I; E, *Eco*RI; B, *Bam*HI; H, *Hind*III). The expanded region of 6 kb represents the region sequenced by Caddle et al. (4) and used to select segments A, B, and C, which were amplified by PCR. The initiation zone identified in this paper by visual inspection of blotting-hybridization data is indicated by the dotted bubble between segments A and C. Quantitative analysis of these data mapped the initiation zone indicated by two shaded areas (B/C and B/A) that overlap (darkly shaded area). Initiation zones in this region identified previously are indicated by shaded bars 1 (2, 3, 2 (9), 3 (1), and 4 (Burhans et al., in press).

estimate was ± 1 sucrose gradient fraction (shaded area in Fig. 3), because all of the ratios one fraction to either side were either greater or less than the designated ratio for the minimum nascent chains. Therefore, the shortest nascent DNA fragments containing segments A and B were between 3 and 7.5 kb and the shortest fragments containing segments C and B were between 4 and 8 kb (Fig. 3). If nascent DNA strands were synthesized bidirectionally, the position of their origin would be 1.5 to 3.7 kb downstream from the 5' end of segment A and 2 to 4 kb upstream of the 3' end of segment C, an initiation zone of about 2.5 kb (Fig. 5). The 1.7-kb overlap (center, shaded) represents the region where most initiation events occurred.

This analysis was based on a symmetrical bidirectional model of replication from the origin. If nascent DNA strands had replicated in only one direction from an origin near segment B, the resulting hybridization patterns for A and C would have been dramatically different from the ones observed (Fig. 1). For example, if replication forks originated near B and then progressed unidirectionally towards C, the shortest nascent DNA chains containing both B and C would be about 3 kb instead of the observed length of 6 kb, and the B to A ratio would remain constant. The coincidence of the two initiation zones defined independently by the B/A and B/C ratios demonstrates that replication from this origin is bidirectional.

DISCUSSION

Initiation of mammalian chromosomal replication has been most extensively studied in the hamster DHFR amplicon (Fig. 5). This sequence consists of approximately 250 kb that includes the DHFR gene and is tandemly repeated about 500 times in CHO 400 cells (8). The DHFR locus initiates replication at the beginning of S phase, and consequently most studies have used CHO 400 cells synchronized at the G_1/S boundary. Initial studies identified specific fragments of

amplified DNA that replicated at the onset of S phase, suggesting that initiation occurred within a 28-kb region downstream from the DHFR gene (8). Improved protocols for labeling and hybridization identified a 4.3-kb DNA fragment as the earliest labeled sequence (2, 3). Further technical improvements ("in-gel renaturation") resolved the position of this initiation zone to about 1.8 kb (9). This initiation zone was refined further by another protocol (1) based on the trioxsalen-cross-linking technique of Russev and Vassilev (12). In this study (1), initiation apparently occurred within a 0.5-kb region, although the presence of repeated sequences introduces ambiguity in the location of this origin (1).

Two potential problems exist in interpreting these results. First, they were carried out exclusively with an amplified sequence that may contain an origin of replication which does not exist in the nonamplified DHFR locus. Second, the cells in these experiments were synchronized at the G_1/S border by isoleucine starvation and aphidicolin inhibition of DNA polymerases, treatments that may alter the process of initiation (5, 6). Recently, a new approach for mapping origins of replication in single copy sequences was developed based on measuring an asymmetric segregation of old prefork histones at replication forks in the absence of new histone synthesis after preincubating cells for 24 h in emetine and BrdU (7). This technique revealed an origin of bidirectional replication within a 15-kb segment that contained the 4.3-kb earliest replicating fragment cited above (Fig. 5). However, the method appears limited in its ability to resolve the position of the origin more precisely, and the mechanism responsible for the observed asymmetry is not clear. Moreover, inhibition of protein biosynthesis for an extended period of time may alter the mechanism for DNA replication.

The method described in this paper can identify a chromosomal origin of DNA replication in mammalian cells containing only a single copy of the origin sequence and proliferating exponentially in the absence of any metabolic inhibitors. Our results revealed that bidirectional DNA replication begins within an initiation zone about 2.5 kb long that is centered approximately 17 kb downstream of the DHFR gene in CHO cells. Initiation within this zone may occur at a unique site or at many sites. However, if initiation occurs at many sites, they must be concentrated near the center of the zone. The location of the DHFR origin mapped by this technique is consistent with results obtained by other mapping techniques (Fig. 5), suggesting that the same sequence or chromosomal segment is utilized as an origin both in normal cells and in cells containing the amplified region. This conclusion was confirmed recently by mapping the sites where discontinuous DNA synthesis changes to continuous DNA synthesis on each strand of this locus that define an origin of bidirectional replication in mechanistic terms (W. C. Burhans, L. T. Vassilev, M. S. Caddle, N. H. Heintz, and M. L. DePamphilis, *Cell*, in press). These transitions occurred within a 0.45-kb segment located within the initiation zone identified here (Fig. 5). The DNA sequence of the earliest replicating region that encompasses the 2.5-kb initiation zone (Fig. 5) has been examined for

structural properties that may be related to origin function (4). Although it contains several candidates for origin-specific sequences, it is premature to draw any conclusions at this time.

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