MOLECULAR AND CELLULAR BIOLOGY, Oct. 1996, p. 5358–5364 Vol. 16, No. 10
0270-7306/96/$04.00+0

High-Resolution Mapping of the Origin of DNA Replication in the Hamster Dihydrofolate Reductase Gene Domain by Competitive PCR

CRISTINA PELIZON, SILVIA DIVIACCO,† ARTURO FALASCHI, AND MAURO GIACCA*

International Centre for Genetic Engineering and Biotechnology, I-34012 Trieste, Italy

Received 13 March 1996/Returned for modification 2 May 1996/Accepted 1 July 1996

By the use of a highly sensitive mapping procedure allowing the identification of the start sites of DNA replication in single-copy genomic regions of untreated, exponentially growing cultured cells (M. Giacca, L. Zentilin, P. Norio, S. Diviacco, D. Dimitrova, G. Contreas, G. Biamonti, G. Perini, F. Weighardt, S. Riva, and A. Falaschi, Proc. Natl. Acad. Sci. USA 91:7119–7123, 1994), the pattern of DNA replication of the Chinese hamster dihydrofolate reductase (DHFR) gene domain was investigated. The method entails the purification of short stretches of nascent DNA issuing from DNA replication origin regions and quantification, within this sample, of the abundance of different adjacent segments by competitive PCR. Distribution of marker abundance peaks around the site from which newly synthesized DNA had emanated. The results obtained by analysis of the genomic region downstream of the DHFR single-copy gene in asynchronous cultures of hamster CHO K1 cells are consistent with the presence of a single start site for DNA replication, located approximately 17 kb downstream of the gene. This site is coincident with the one detected by other studies using different techniques in CHO cell lines containing an amplified DHFR gene domain.

In mammalian cells, long-standing evidence indicates that DNA in each chromosome is synthesized by multiple tandemly organized replication units (replicons), which are activated at precisely defined times of the S phase (27). By analogy with prokaryotic, animal virus, and yeast chromosomes, it is assumed that initiation of DNA replication in each replicon occurs at specific sites (origins of replication) from which two oppositely moving semiconservative forks originate (for reviews, see references 10 and 17). However, despite massive efforts in the last several years, the identification and characterization of the actual DNA sequences where replication initiates in mammalian cells, and of the *cis*-acting proteins interacting with these sequences, remain elusive.

The identification of DNA replication origins in mammalian cells has been mainly hampered by the lack of a specific plasmid-based replication assay (2) similar to the one that proved successful in studies of yeast cells (38). Autonomous replication of plasmids containing putative origin sequences either has been unsuccessful or unreproducible (5, 6, 21) or has proven to be nonspecific (26, 30, 31).

To circumvent this problem, a number of mapping approaches have been developed to identify origins directly on the chromosomes. These involve the analysis of distribution of nascent DNA (1, 20, 40, 41), the identification of the region where the transition from discontinuous to continuous DNA synthesis occurs (5, 24, 29), or the analysis of the replication intermediates by two-dimensional (2-D) gel electrophoresis (34, 42).

The overall difficulty of the above-described approaches for origin identification, despite their diversity, derives from the fact that they must deal with an extremely limited amount of origin-specific DNA dispersed in a huge amount of unspecific chromosomal DNA. To overcome this problem, one of the most studied regions has been the dihydrofolate reductase (DHFR) gene domain in the CHOC 400 cell line. This cell line is a derivative of the Chinese hamster ovary (CHO) K1 cell line in which the DHFR locus is amplified over 1,000-fold (35). Initial studies on synchronized CHOC 400 cells identified the presence of a DNA replication initiation region downstream of the DHFR gene (25). Subsequent higher-resolution methods mapped an origin of replication (termed ori-β) to an approximately 5-kb region positioned ~17 kb downstream of the DHFR gene (4); this area was later narrowed to an approximately 2-kb DNA subfragment (32). Finally, results from studies using a cross-linking strategy suggested that an initiation site may be contained within an approximately 500-bp subfragment of the previously defined earliest-labeled fragment (1).

Also, the DNA replication pattern of the single-copy gene locus in the parental CHO K1 cells has been studied through the analysis of polarity of leading-strand synthesis (24) and of Okazaki fragments (5). While the former study gave low-resolution results grossly overlapping those obtained with the amplified gene domain, the latter indicated that DNA replication initiates within a 450-bp segment of the earliest-labeled fragment but differing from the one previously identified by cross-linking of nascent DNA. The same region was also identified by a semiquantitative study using PCR amplification of nascent DNA (41).

Taken together, these observations point to the existence of a precise initiation region in the DHFR locus and are in apparent contradiction with the results obtained by the study of the replication intermediates in this region by the 2-D gel electrophoresis approach. This method, that has been successfully used in a number of studies on viral and yeast replicons, indicated that initiation of replication occurs at many sites within a 50-kb area downstream of the DHFR gene, either in the amplified (11, 13, 14, 42) or in the single-copy (12) gene locus.

The reasons for the discrepancy between the results ob-

* Corresponding author. Mailing address: ICGEB, Padriciano 99, 34012 Trieste, Italy. Phone: 39-40-3757.324. Fax: 39-40-226555. Electronic mail address: giacca@icgeb.trieste.it.

† Present address: Laboratoire de Biophysique, Muséum National d'Histoire Naturelle, INSERM Unité 201-CNRS UA 481, 75231 Paris Cedex 05, France.

tained by the 2-D gel mapping technique (origin dispersed within a broad initiation zone) and those obtained by the analysis of nascent DNA length or polarity (origin circumscribed within a small genomic region) are not understood. Some hypotheses have been put forward to reconcile these opposite findings (10, 23, 33), but no proof for any of them has been presented. However, most of the studies performed so far entail the use of potentially artifactual techniques (such as treatment with protein inhibitors, cell synchronization, or cell permeabilization) or produce results which are often difficult to interpret (such as 2-D gel patterns of migration).

To avoid any potential artifact in the procedure for origin mapping, we have developed a technique which can be applied to exponentially growing cells without the use of any metabolic inhibitor and to single-copy genomic loci. The procedure is based on the quantification of different markers along a given genomic region in samples of short, newly synthesized DNA (3). According to the functional definition of an origin of DNA replication, newly replicated DNA issues bidirectionally from the origin and progressively increases in length, covering adjacent sequences on the DNA. As a consequence, the relative abundance of defined markers within a given genomic area in samples of short nascent DNA fragments is inversely correlated with their distance from the origin, giving a distribution of values peaking at the replication start site.

Using competitive PCR quantification of nascent DNA strands in synchronized and asynchronously growing human HL-60 cells, we could precisely map the localization of an origin of DNA replication within a 474-bp segment in the lamin B2 gene domain (20). In this work, the same procedure was used for a high-resolution mapping study on the single-copy DHFR gene locus in hamster CHO K1 cells.

MATERIALS AND METHODS

Cell cultures and DNA labeling. CHO K1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 50 μg of gentamicin per ml. Cells (2×10^8) were pulse-labeled for 15 min at 37°C with 1 μM (final concentration) [^{3}H]deoxycytidine (21.5 Ci/mmol; Amersham) and 100 μM (final concentration) unlabeled 5′-bromodeoxyuridine (BrdUrd; Boehringer Mannheim GmbH, Mannheim, Germany) as described previously (20).

DNA sequencing. A segment of the genomic CHO K1 cell DNA in the DHFR genomic region (nucleotides [nt] 1500 to 3210 of the sequence *cgdhfrori* in GenBank) was sequenced by the dideoxy-chain termination method (T7 Sequencing kit; Pharmacia Biotech). Primers chosen in this region were constructed according to the sequence obtained, which, in several instances, was diverging from the published sequence (7).

Extraction and purification of newly synthesized DNA. Total DNA from pulse-labeled cells was extracted from purified nuclei (final volume of 1 ml) alkali denatured, and size fractionated by sedimentation through two 5 to 30% neutral sucrose gradients (35 ml each) for 18 h at 20°C in a Beckman SW28 rotor at 26,000 rpm. Thirty-three fractions of 1 ml each were collected from the top of the gradients. Two fractions containing DNA fragments of approximately 1,000 nt were selected, dialyzed, further purified by anti-BrdUrd immunoaffinity chromatography (9), and analyzed by competitive PCR (16). The same procedure was adopted for three other fractions flanking the preceding ones that were pooled, purified, and used for quantitative PCR experiments.

Primer choice and competitor construction. Seven sets of four primers each were constructed in the hamster DHFR region according to the nucleotide sequence determined. The locations of these primer sets are shown in Fig. 1, and their nucleotide sequences are given in Table 1. Each primer set consists of two oligonucleotides used for PCR amplification (external primers [the first two of each set in Table 1]) and two oligonucleotides used only for competitor construction (internal primers). The latter two primers consist of two common 5′ tails of 20 nt (tail1 [5′-ACCTGCAGGGATCCGTCGAC-3′] and tail2 [5′-GTC GACGGATCCCTGCAGGT-3′]) linked to specific sequences complementary to the respective genomic targets at their 3′ ends.

Competitors for competitive PCR experiments consisted of the amplification products obtained by the two external primers with the addition of a 20-bp unrelated sequence in the middle, in order to allow identification of genomic target and competitor PCR products by polyacrylamide gel electrophoresis. The method for competitor construction is described in detail elsewhere (16, 22).

Quantification of the competitor DNA fragments was obtained by coamplification of scalar amounts (10-fold dilutions, followed by progressively closer dilutions in the range of equivalence) of competitor with a fixed amount of total genomic DNA of CHO K1 cells. Competitors were then directly used in competitive PCR assays on nascent DNA.

Competitive PCR experiments. Quantification of the abundance of the seven DNA segments amplified with the chosen primer sets was obtained in samples of nascent DNA by competitive PCR. The experiments were carried out by mixing a fixed quantity of BrdUrd DNA (5 μl of each preparation) with scalar quantities of competitor DNA fragments. Rough quantitations were obtained by 10-fold dilutions; precise quantitations were then obtained by using competitor dilutions in the equivalence range.

Conditions for PCR amplification were as follows: denaturation at 94°C for 30 s; annealing at the temperatures reported in Table 1 for each primer set for 30 s; and extension at 72°C for 30 s. Fifty cycles of amplification were performed for each amplification experiment with 1 U of *Taq* polymerase (Perkin-Elmer, Emeryville, Calif.), using the conditions recommended by the manufacturer.

Nucleotide sequence accession number. The updated nucleotide sequence, obtained in collaboration with E. Fanning and A. Schmidt, is available in the EMBL nucleotide sequence data base (accession number X94372).

RESULTS

A mapping procedure based on the precise quantification of the abundance of different DNA segments scattered along the genomic region of interest in samples of nascent DNA (20) was applied to the single-copy DHFR gene domain in order to localize an origin of DNA replication. For this purpose, exponentially growing CHO K1 cells were pulse-labeled for 15 min with [^{3}H]deoxycytidine and BrdUrd. During this period of time, stretches of newly synthesized DNA deriving from all portions of the genome (and thus at different distances from origins of DNA replication) were labeled. Subsequently, cells were collected, and total genomic DNA was extracted and denatured by alkali treatment. Within this sample, short stretches of single-stranded newly synthesized DNA (i.e., nascent DNA which is the closest to the origin) were size selected by sedimentation through neutral 5 to 30% sucrose gradients. Fractions with an average size of ~1,000 nt were collected as representative of nascent DNA emanating from all origins of DNA replication of the genome. In our experience, this size is optimal for avoiding contamination by Okazaki fragments (which are shorter) or contamination by randomly broken genomic DNA (which is likely to be larger). Additionally, this average length of nascent DNA is optimal for PCR amplification, the sizes of the chosen amplification products being 150 to 300 bp, and gives a satisfactory resolution among closely spaced genomic segments, contrary to the results obtained with longer fragments.

Further purification of newly synthesized, BrdUrd-substituted DNA was obtained by batch chromatography with an affinity resin bearing anti-BrdUrd antibodies as described previously (9). This final purification step is used to completely eliminate any trace of parental, unlabeled DNA possibly contaminating the preparation by random breakage during the extraction procedure.

With these samples of nascent DNA, competitive PCR experiments for origin mapping were performed by the analysis of seven segments in the DHFR locus. Since we initially found that several chosen primer pairs in this region were not amplifying from total CHO DNA, the nucleotide sequence of a DNA region downstream of the DHFR gene (corresponding to nt 1500 to 3210 of the sequence *cgdhfrori* in GenBank) was redetermined by the analysis of overlapping PCR products. The new nucleotide sequence was found to differ in several positions from the published sequence; therefore, the sequences used for the synthesis of PCR primers were chosen on the basis of the newly determined sequence.

Seven primer sets were synthesized (Table 1); one is located within the 3′ nontranslated region of the DHFR gene, while the other six are positioned in a ~6-kb region (7) located ~15

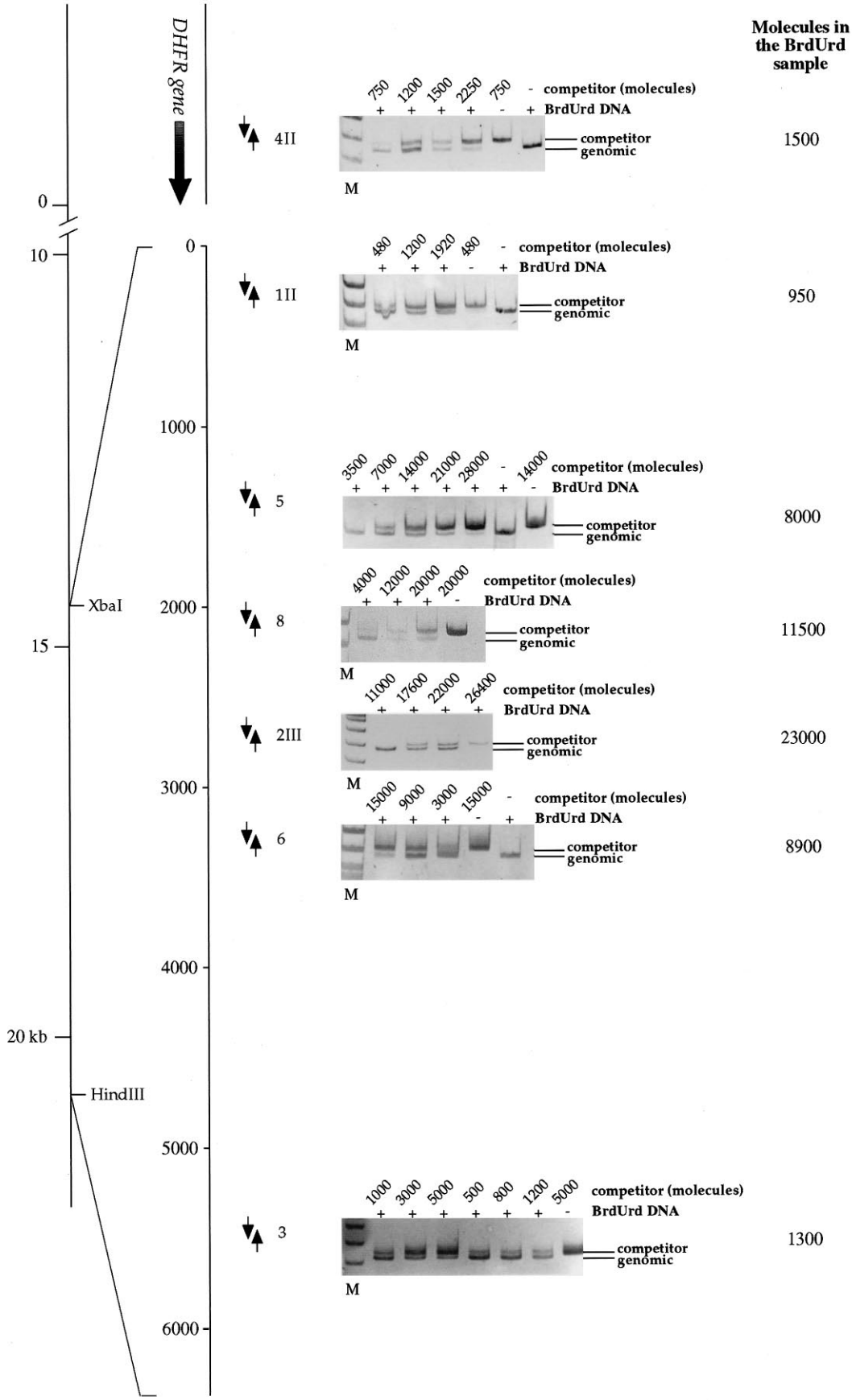
DHFR gene
0
10
15
20 kb
XbaI
HindIII
0
1000
2000
3000
4000
5000
6000
4II
1III
5
8
2III
6
3
Molecules in the BrdUrd sample
competitor (molecules)
BrdUrd DNA
competitor
genomic
M
750 1200 1500 2250 750 - competitor (molecules)
+ + + + - + BrdUrd DNA
1500
480 1200 1920 480 - competitor (molecules)
+ + + - + BrdUrd DNA
950
3500 7000 14000 21000 28000 - 14000 competitor (molecules)
+ + + + + + - BrdUrd DNA
8000
4000 12000 20000 20000 competitor (molecules)
+ + + - BrdUrd DNA
11500
11000 17600 22000 26400 competitor (molecules)
+ + + + BrdUrd DNA
23000
15000 9000 3000 15000 - competitor (molecules)
+ + + - + BrdUrd DNA
8900
1000 3000 5000 500 800 1200 5000 competitor (molecules)
+ + + + + + - BrdUrd DNA
1300

FIG. 1. Competitive PCR mapping of the DNA replication origin in the DHFR locus. A schematic representation of the genomic area downstream of the Chinese hamster DHFR gene is shown at the left. In this area, six primer pairs were selected within a genomic region located ~15 kb downstream of the gene, and one primer pair was selected within the 3′ end of the gene itself. The locations of these primers are indicated by converging arrows, and their nucleotide sequences are presented in Table 1. For each primer pair, competitor DNA fragments for quantitative PCR were constructed and quantified as described previously (16, 22). These competitors contain exactly the same sequence as the target genomic DNA except for a 20-bp insertion in the middle to allow resolution by polyacrylamide gel electrophoresis. The results of a competitive PCR experiment for this region are shown on the right. Quantitation of the abundance of the different markers in a sample of nascent, BrdUrd-substituted DNA was achieved by coamplification of a fixed amount of sample with increasing concentrations of competitor, as indicated above each gel. Since the amount of competitor added to the reaction is known, the amount of target genomic DNA can be easily derived from the ratio between the two final amplification products, as determined by densitometric scanning (16). For each gel, the competitor and genomic DNA amplification products are indicated. The results of the quantitations obtained for this sample of BrdUrd DNA are indicated by the values reported on the right. M, molecular weight marker. Numbering is according to the sequence *cgdhfrori* in GenBank.

kb downstream of the gene (Fig. 1). For each primer set, we constructed a competitor DNA fragment containing exactly the same sequence as the target genomic DNA segment except for a 20-bp insertion in the middle to allow resolution of the genomic and competitor amplification products by polyacrylamide gel electrophoresis. These competitors were constructed by a recombinant PCR procedure starting directly from the genomic amplification products as already described (16). The sequences of the internal primers used for the generation of these competitors, bearing at their 5′ ends 20-nt tails complementary to each other which subsequently become the 20-bp insertion tags of the competitors, are given in Table 1.

Results of a competitive PCR experiment are shown in Fig. 1. A fixed amount of nascent DNA was mixed with increasing amounts of competitor for each primer set and amplified. The PCR products were resolved by gel electrophoresis, and the intensity of each band (genomic and competitor) was determined by densitometric scanning. According to the principles of competitive PCR, the ratio between the two molecular species remains unchanged during the amplification process. Since the number of added competitor molecules is known, and the final ratio between the PCR products is measured, the initial amount of target DNA can be calculated in a simple way. These measurements are given at the right in Fig. 1.

The average results of the quantifications obtained by different experiments are shown in Fig. 2. At least four independent competitive PCR quantifications were performed for each primer set by using three different nascent DNA fragment preparations. The results obtained by these analyses revealed a distribution of abundance of genomic markers in the DHFR genomic region, peaking at the segment amplified by primer set 2III. This segment is enriched over 10-fold with respect to those localized at the two boundaries of the analyzed region (primer sets 1II and 3) or within the 3′ end of DHFR gene itself (primer set 4II), which is approximately 17 kb away.

From the distance between the two DNA segments (amplified by primer sets 8 and 6) flanking the segment amplified by primer set 2III, it can be concluded that a start site for DNA

TABLE 1. Primer sequences and PCR parameters[a]

Primer set	Primer name	Location (nt)	Sequence	Size (bp) of PCR product	Annealing temp (°C)
1II	1DXII	377–358	5′-AAAAGCCACTCAAGCGCCAA-3′		
	1SX	153–172	5′-GGAAGGATTGTAGGAGCCAA-3′	225	54
	1PERDXII	275–294	5′-tail1+CAACCTAGGAACTCTGCATG-3′		
	1PERSXII	274–255	5′-tail2+GCCTCATGCAGTTCCCTAAT-3′		
2III	2DXIII	2879–2860	5′-CCTTCATGCTGACATTTGTC-3′		
	2SXIII	2602–2621	5′-GTCCCTGCCTCAAAACACAA-3′	278	64
	2PERDXIII	2679–2698	5′-tail1+CTCAGTGAGTCCACTTCTTT-3′		
	2PERSXIII	2678–2659	5′-tail2+AAGGAAGGAAAGAAAGGCCC-3′		
3	3DX	5652–5633	5′-GCTGGGATAAGTTGAAATCC-3′		
	3SX	5394–5416	5′-GGACACTAAGTCTAGGTACTACA-3′	259	54
	3PERDX	5524–5543	5′-tail1+AGGACTCAGCTCTTACTAAC-3′		
	3PERSX	5523–5504	5′-tail2+TAGGAAACTGAGATGCCAGG-3′		
4II	4DX	1898–1877	5′-TATGGGCTACCTCCTTAGGAGC-3′		
	4SXII	1686–1705	5′-CCAGTGATATGCATAGCACC-3′	213	60
	4PERDX	1821–1840	5′-tail1+CCATCCAAGAGCAATGGCAA-3′		
	4PERSX	1820–1801	5′-tail2+TCACCACAGCTAGATGGTAA-3′		
5	5DX	1546–1527	5′-GAGCTAGGAGGATCCATTCT-3′		
	5SX	1337–1356	5′-GAGACGAGGGATTTACTCTA-3′	210	60
	5PERDX	1438–1457	5′-tail1+TGGAATGCTCTCTCTAGCTT-3′		
	5PERSX	1437–1418	5′-tail2+TGAACACAGACACTATGGTC-3′		
6	6DX	3210–3191	5′-AACCTCTGAACTGTAAGCTG-3′		
	6SXbis	3041–3061	5′-GAACTGGCTTCCCAAGAAAT-3′	169	62
	6PERDX	3133–3151	5′-tail1+TGCTGTGAAGAGACACCATG-3′		
	6PERSX	3132–3113	5′-tail2+ATAGAAACCCCAGCTAAGAC-3′		
8	8DX	2205–2186	5′-GTCCTCGGTATTAGTTCTCC-3′		
	8SXII	2002–2021	5′-CTCTCTCATAGTTCTCAGGC-3′	204	56
	8PERDX	2081–2100	5′-tail1+TCCATGGCAGTCTTCACACT-3′		
	8PERSX	2080–2061	5′-tail2+CATTCATCAAGCTGGAAAGC-3′		

[a] The first two primers of each set were used for amplification from CHO genomic DNA; their locations are schematically shown in Fig. 1. The last two primers of each set were used for competitor construction. The sequences of tail1 and tail2 are reported in Materials and Methods. Nucleotide numbering is according to the sequence *cgdhfrori* in GenBank (although the actual nucleotide sequences are according to the new sequencing data obtained for CHO K1 cells) for all primer sets except 4II, whose nucleotide numbering is according to the sequence *crudhfraa* in GenBank.

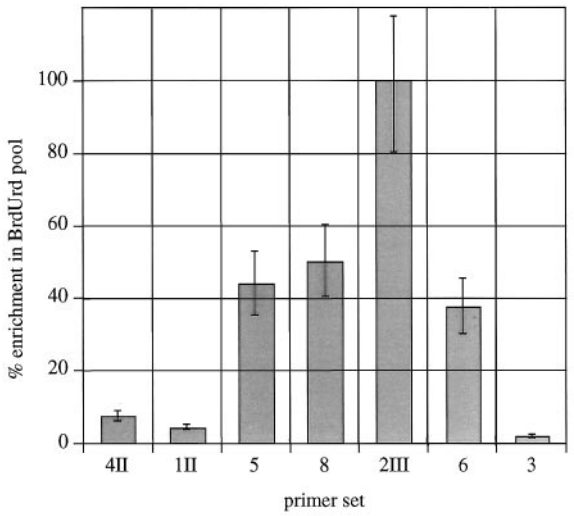

FIG. 2. Quantitation of abundance of the analyzed genomic segments of Fig. 1 in newly synthesized DNA samples of ~1,000 nt. The results are expressed as a percentage of enrichment for each segment with respect to the most abundant one (the segment detected by primer set 2III). The results are the average of at least four independent determinations for each segment, obtained by using three nascent DNA preparations; the bars indicate standard deviations. A clear enrichment for the segment amplified by primer set 2III is evident, thus indicating that a start site for DNA replication is located in an ~800-bp region located between primer sets 8 and 6.

replication is located within an approximately 800-bp region centered over fragment 2III (Fig. 3).

DISCUSSION

The DHFR gene domain is the most thoroughly investigated mammalian origin of DNA replication. Replication studies of this region have been initially facilitated by using a cell line in which the DHFR gene is amplified 1,000-fold (CHOC 400) and subsequently extended to the analysis of the single-copy gene by using procedures that synchronize cells at the G_1/S boundary. However, conflicting results have been obtained for this region with respect to the location of the replication origin. These results range from origin localization in a 450-bp stretch on the basis of analysis of the Okazaki fragment polarity switch (5) to localization in a 50-kb region in studies using the 2-D gel technique (11, 13, 42). While it is difficult to reconcile these results, it should be pointed out that the vast majority of studies analyzing newly synthesized DNA emanating from replication start sites (by analysis either of its distribution along the chromosome or of its polarity) have led to the identification of precise sites for initiation of DNA replication. Among the identified origins are those located within the ribosomal protein S14 gene in Chinese hamster cells (39), within the Syrian hamster *CAD* gene (28), and near the mouse adenosine deaminase gene (8, 43), the Chinese hamster rhodopsin gene (19), the human β-globin gene (29), the c-*myc* gene (40), and the human lamin B2 gene (20). In contrast, most of the studies analyzing the structure of replication intermediates by the 2D-gel technique led to the conclusion that origins are dispersed in wide genomic areas or, at best, confined to still large preferred initiation zones (11, 32, 34, 42). Therefore, it seems that all of these conclusions could be biased by the method used.

To avoid any possible artifact deriving from the experimental technique, to analyze the behavior of single-copy gene domains, and to achieve sufficient resolution to allow subsequent protein-DNA interaction studies, it seemed important to develop an assay that could be performed in physiological conditions and by using the simplest and least artifact-prone procedure. All of these demands are satisfied by the mapping procedure by competitive PCR quantification of short stretches of nascent DNA which can be applied to single-copy genes, does not require synchronization or other DNA metabolism-altering procedures, and has a high resolution capacity (20). We have also recently demonstrated that even cell labeling with BrdUrd and subsequent purification of BrdUrd-substituted DNA fragments can be omitted from this procedure without changes in the final results (31a). This observation definitely rules out the possibility of the introduction of a bias in fragment selection on the anti-BrdUrd column or of artifactual DNA fragmentation by photodamage of BrdUrd-substituted DNA. However, the

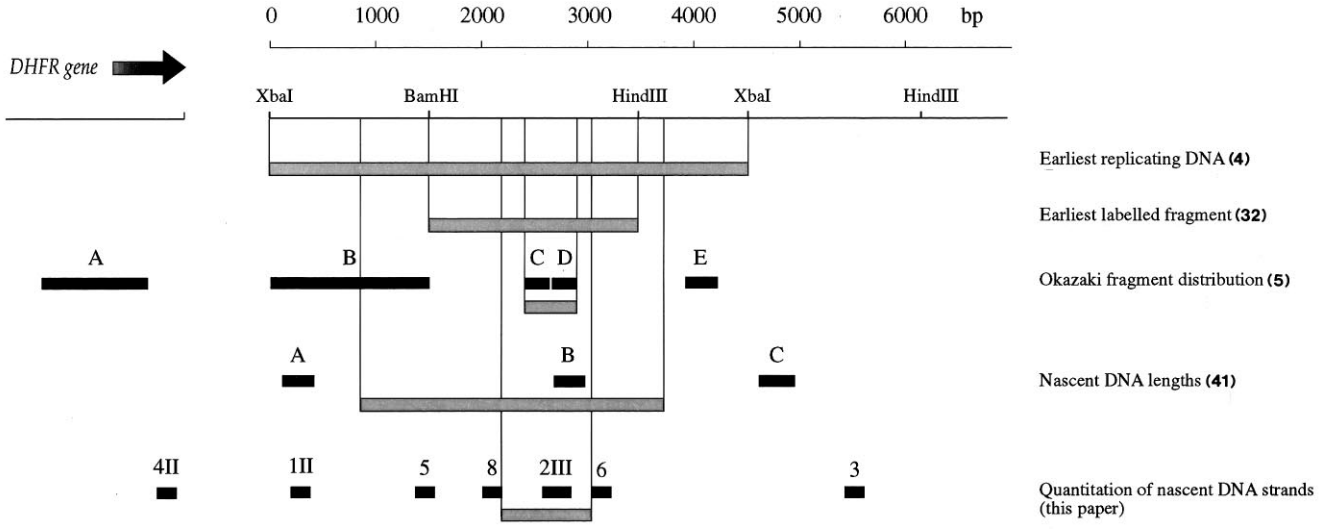

FIG. 3. Localization of the start site for DNA replication identified by quantitation of nascent DNA via competitive PCR and by different methods applied to CHOC 400 or CHO K1 cells. A segment of ~6 kb located approximately 15 kb downstream of the DHFR gene is represented as a schematic restriction map. Probes used to determine the location of the origin region in different studies are indicated as solid bars. The origin regions defined by the use of these probes are indicated by gray bars.

finding that BrdUrd labeling can be omitted is not surprising, since the procedure developed for origin identification in asynchronous cells relies in principle only on the selection of nascent DNA stretches according to their sizes.

The application of this method to the single-copy DHFR locus of CHO K1 cells leads to the conclusion that replication starts at a defined region of ~800 bp (from nt 2206 to 3040 in the region sequenced by Caddle et al. [7]) located approximately 17 kb downstream of the gene. This site is coincident with the one detected by the analysis of Okazaki fragment polarity (5), which, in turn, is in agreement with most of the other studies analyzing the DNA replication pattern of the amplified domain (Fig. 3). It is very unlikely that the distribution of genomic marker abundance found in the analyzed region and presented in Fig. 2 is representative of an initiation zone dispersed between markers 5 and 6 (<2,000 bp), with a preferred initiation site around marker 2III. In fact, the distribution found resembles the one expected from the gaussian distribution of the nascent DNA fragments of ~1,000 nt obtained by sucrose gradient purification.

Analysis of the representation of different markers in the DHFR region in purified nascent DNA strands of increasing length was adopted also by Vassilev et al., who used conventional PCR (41). However, this method, although exploiting the extraordinary sensitivity of PCR, is subjected to all of the uncontrollable variables affecting the quantitative outcome of conventional PCR (16, 18, 37). On the other hand, quantitative PCR using internal competitive DNA templates (16, 36) should be considered one of the techniques of choice for precise quantitation of low abundance nucleic acids using PCR (37).

A further important advantage of origin mapping by competitive PCR of nascent DNA is that this technique, unlike other methods (24), is able to identify origins at relatively high resolution. High-resolution mapping is required to study the protein-DNA interactions underlying the origin activation event by using in vivo footprinting methods (15).

In conclusion, the results presented in this work reinforce the notion that DNA replication starts at a defined site downstream of the DHFR gene in Chinese hamster cells. The longstanding discrepancy between this conclusion and the one derived from 2-D gel analysis remains to be understood.

ACKNOWLEDGMENTS

This work was partially supported by grant ERBCHRXCT920022 from the Human Capital and Mobility Program of the European Community. C.P. is supported by an SISSA (Trieste) predoctoral fellowship.

REFERENCES

1. **Anachkova, B., and J. L. Hamlin.** 1989. Replication in the amplified dihydrofolate reductase domain in CHO cells may initiate at two distinct sites, one of which is a repetitive sequence element. Mol. Cell. Biol. **9:**532–540.
2. **Biamonti, G., G. Della Valle, D. Talarico, F. Cobianchi, S. Riva, and A. Falaschi.** 1985. Fate of exogenous recombinant plasmids introduced into mouse and human cells. Nucleic Acids Res. **13:**5545–5561.
3. **Biamonti, G., G. Perini, F. Weighardt, S. Riva, M. Giacca, P. Norio, L. Zentilin, S. Diviacco, D. Dimitrova, and A. Falaschi.** 1992. A human DNA replication origin: localization and transcriptional characterization. Chromosoma **102:**S24–S31.
4. **Burhans, W. C., J. E. Selegue, and N. H. Heintz.** 1986. Isolation of the origin of replication associated with the amplified Chinese hamster dihydrofolate reductase domain. Proc. Natl. Acad. Sci. USA **83:**7790–7794.
5. **Burhans, W. C., L. T. Vassilev, M. S. Caddle, N. H. Heintz, and M. L. DePamphilis.** 1990. Identification of an origin of bidirectional DNA replication in mammalian chromosomes. Cell **62:**955–965.
6. **Caddle, M. S., and M. P. Calos.** 1992. Analysis of the autonomous replication behavior in human cells of the dihydrofolate reductase putative chromosomal origin of replication. Nucleic Acids Res. **20:**5971–5978.
7. **Caddle, M. S., R. H. Lussier, and N. H. Heintz.** 1990. Intramolecular DNA triplexes, bent DNA and DNA unwinding elements in the initiation region of an amplified dihydrofolate reductase replicon. J. Mol. Biol. **211:**19–33.
8. **Carroll, S. M., M. L. DeRose, J. L. Kolman, G. H. Nonet, R. E. Kelly, and G. M. Wahl.** 1993. Localization of a bidirectional DNA replication origin in the native locus and in episomally amplified murine adenosine deaminase loci. Mol. Cell. Biol. **13:**2971–2981.
9. **Contreas, G., M. Giacca, and A. Falaschi.** 1992. Purification of BrdUrd-substituted DNA by immunoaffinity chromatography with anti-BrdUrd antibodies. BioTechniques **12:**824.
10. **DePamphilis, M. L.** 1993. Eukaryotic DNA replication: anatomy of an origin. Annu. Rev. Biochem. **62:**29–63.
11. **Dijkwel, P. A., and J. L. Hamlin.** 1992. Initiation of DNA replication in the dihydrofolate reductase locus is confined to the early S-period in CHO cells synchronized with the plant amino acid mimosine. Mol. Cell. Biol. **12:**3715–3722.
12. **Dijkwel, P. A., and J. L. Hamlin.** 1995. The Chinese hamster dihydrofolate reductase origin consists of multiple potential nascent-strand start sites. Mol. Cell. Biol. **15:**3023–3031.
13. **Dijkwel, P. A., J. P. Vaughn, and J. L. Hamlin.** 1991. Mapping replication initiation sites in mammalian genomes by two-dimensional gel analysis: stabilization and enrichment of replication intermediates by isolation on the nuclear matrix. Mol. Cell. Biol. **11:**3850–3859.
14. **Dijkwel, P. A., J. P. Vaughn, and J. L. Hamlin.** 1994. Replication initiation sites are distributed widely in the amplified CHO dihydrofolate reductase domain. Nucleic Acids Res. **22:**4989–4996.
15. **Dimitrova, D., M. Giacca, F. Demarchi, G. Biamonti, S. Riva, and A. Falaschi.** 1996. In vivo protein-DNA interactions at a human DNA replication origin. Proc. Natl. Acad. Sci. USA **93:**1498–1503.
16. **Diviacco, S., P. Norio, L. Zentilin, S. Menzo, M. Clementi, G. Biamonti, S. Riva, A. Falaschi, and M. Giacca.** 1992. A novel procedure for quantitative polymerase chain reaction by coamplification of competitive templates. Gene **122:**313–320.
17. **Falaschi, A., and M. Giacca.** 1994. The quest for a human *ori*. Genetica **94:**255–266.
18. **Ferre, F.** 1992. Quantitative or semi-quantitative PCR: reality versus myth. PCR Methods Appl. **2:**1–9.
19. **Gale, J. M., R. A. Tobey, and J. A. D'Anna.** 1992. Localization and DNA sequence of a replication origin in the rhodopsin gene locus of Chinese hamster cells. J. Mol. Biol. **224:**343–358.
20. **Giacca, M., L. Zentilin, P. Norio, S. Diviacco, D. Dimitrova, G. Contreas, G. Biamonti, G. Perini, F. Weighardt, S. Riva, and A. Falaschi.** 1994. Fine mapping of a replication origin of human DNA. Proc. Natl. Acad. Sci. USA **91:**7119–7123.
21. **Gilbert, D., and S. N. Cohen.** 1989. Autonomous replication in mouse cells: a correction. Cell. **56:**143–144.
22. **Grassi, G., L. Zentilin, S. Tafuro, S. Diviacco, A. Ventura, and M. Giacca.** 1994. A rapid procedure for the quantitation of low abundance mRNAs by competitive RT-PCR. Nucleic Acids Res. **22:**4547–4549.
23. **Hamlin, J. L., and P. A. Dijkwel.** 1995. On the nature of replication origins in higher eukaryotes. Curr. Opin. Genet. Dev. **5:**153–161.
24. **Handeli, S., A. Klar, M. Meuth, and H. Cedar.** 1989. Mapping replication units in animal cells. Cell **57:**909–920.
25. **Heintz, N. H., and J. L. Hamlin.** 1982. An amplified chromosomal sequence that includes the gene for dihydrofolate reductase initiates replication within specific restriction fragments. Proc. Natl. Acad. Sci. USA **79:**4083–4087.
26. **Heinzel, S. S., P. J. Krysan, C. T. Tran, and M. P. Calos.** 1991. Autonomous DNA replication in human cells is affected by the size and the source of the DNA. Mol. Cell. Biol. **11:**2263–2272.
27. **Huberman, J. A., and A. D. Riggs.** 1968. On the mechanism of DNA replication in mammalian chromosomes. J. Mol. Biol. **32:**327–337.
28. **Kelly, R. E., M. L. DeRose, B. W. Draper, and G. M. Wahl.** 1995. Identification of an origin of bidirectional DNA replication in the ubiquitously expressed mammalian *CAD* gene. Mol. Cell. Biol. **15:**4136–4148.
29. **Kitsberg, D., S. Selig, J. Keshet, and H. Cedar.** 1993. Replication structure of the human β-globin gene domain. Nature (London) **368:**588–590.
30. **Krysan, P. J., and M. P. Calos.** 1991. Replication initiates at multiple locations on an autonomously replicating plasmid in human cells. Mol. Cell. Biol. **11:**1464–1472.
31. **Krysan, P. J., S. B. Haase, and M. P. Calos.** 1989. Isolation of human sequences that replicate autonomously in human cells. Mol. Cell. Biol. **9:**1026–1033.

31a. **Kumar, S., M. Giacca, P. Notio, G. Biamonti, S. Riva, and A. Falaschi.** Utilization of the same DNA replication origin by human cells of different derivation. Nucleic Acids Res., in press.

32. **Leu, T.-H., and J. L. Hamlin.** 1989. High-resolution mapping of replication fork movement through the amplified dihydrofolate reductase domain in CHO cells by in-gel renaturation analysis. Mol. Cell. Biol. **9:**523–531.
33. **Linskens, M. H., and J. A. Huberman.** 1990. The two faces of higher eukaryotic DNA replication origins. Cell **62:**845–847.
34. **Little, R. D., T. H. K. Platt, and C. L. Schildkraut.** 1993. Initiation and

termination of DNA replication in human rRNA genes. Mol. Cell. Biol. **13:**6600–6613.
35. **Milbrandt, J. D., N. H. Heintz, W. C. White, S. M. Rothman, and J. L. Hamlin.** 1981. Methotrexate-resistant Chinese hamster ovary cells have amplified a 135-kilobase-pair region that includes the dihydrofolate reductase gene. Proc. Natl. Acad. Sci. USA **78:**6043–6047.
36. **Sestini, R., C. Orlando, L. Zentilin, S. Gelmini, P. Pinzani, M. Giacca, and M. Pazzagli.** 1994. Measuring c-*erb*B-2 oncogene amplification in fresh and paraffin-embedded tumors by competitive polymerase chain reaction. Clin. Chem. **40:**630–636.
37. **Siebert, P. D., and J. W. Larrick.** 1992. Competitive PCR. Nature (London) **359:**557–558.
38. **Stinchcomb, D. T., K. Struhl, and R. W. Davis.** 1979. Isolation and characterization of a yeast chromosomal replicator. Nature (London) **282:**39–43.
39. **Tasheva, E. S., and D. J. Roufa.** 1994. A mammalian origin of bidirectional DNA replication within the Chinese hamster RPS14 locus. Mol. Cell. Biol. **14:**5628–5635.
40. **Vassilev, L., and E. M. Johnson.** 1990. An initiation zone of chromosomal DNA replication located upstream of the c-*myc* gene in proliferating HeLa cells. Mol. Cell. Biol. **10:**4899–4904.
41. **Vassilev, L. T., W. C. Burhans, and M. L. DePamphilis.** 1990. Mapping an origin of DNA replication at a single-copy locus in exponentially proliferating mammalian cells. Mol. Cell. Biol. **10:**4685–4689.
42. **Vaughn, J. P., P. A. Dijkwel, and J. L. Hamlin.** 1990. Replication initiates in a broad zone in the amplified CHO dihydrofolate reductase domain. Cell **61:**1075–1087.
43. **Virta-Pearlman, V. J., P. H. Gunaratne, and A. C. Chinault.** 1993. Analysis of a replication initiation sequence from the adenosine deaminase region of the mouse genome. Mol. Cell. Biol. **13:**5931–5942.