Journal of Cellular Biochemistry 85:346–356 (2002)

Mapping of an Origin of DNA Replication Near the Transcriptional Promoter of the Human HPRT Gene

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Abstract A quantitative PCR method was used to map a functional origin of DNA replication in the hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene in normal human fibroblasts. This PCR method measures the abundance of specific sequences in short fragments of newly replicated DNA from logarithmically growing cells. Quantitative measurements rely on synthetic molecules (competitors) that amplify with the same primer sets as the target molecules, but generate products of different sizes. This method was first utilized to determine the position of the replication origin near the lamin B2 gene (Giacca et al. [1994] Proc. Natl. Acad. Sci. U S A. 91:7119–7123). In the present study, primer sets were tested along a 16-kb region near exon 1 of the HPRT gene. The most abundant fragment was found to be located in the first intron of HPRT, just downstream of the promoter and exon 1 of the gene, and approximately 3.5 kb upstream of a previously reported autonomously replicating sequence (Sykes et al. [1988] Mol. Gen. Genet. 212:301–309). J. Cell. Biochem. 85: 346–356, 2002. © 2002 Wiley-Liss, Inc.

Key words: replication origin; ARS; HPRT; X chromosome; quantitative PCR

DNA replication in eukaryotic cells is a highly organized and well-controlled process. Once per S phase, initiation of DNA synthesis occurs at a centrally located origin within replication units called replicons. Replicons are heterogeneous in size, and according to a recent report [Berezney et al., 2000], have an average length that is likely to be greater than 100 kb. There are an estimated 30,000 origins of DNA replication in a mammalian cell [Todorovic et al., 1999], but few of these have been identified. In recent years, a new technique has been used by several groups to characterize novel origins of replication. Short fragments (1–2 kb in length) of newly-replicated DNA are isolated from asynchronous cells and the relative abundance of a sequence of interest is then determined using quantitative competitive PCR. Origins mapped using this technique include those in the human lamin B2 [Giacca et al., 1994], β-globin [Aladjem et al., 1995], c-myc [Tao et al., 2000], and DNA methyl transferase (DNMT1) [Araujo et al., 1999] genes, as well as ori-β and ori-β′ in the hamster dihydrofolate reductase (DHFR) gene [Pelizon et al., 1996; Kobayashi et al., 1998]. A compilation of the few known mammalian origins and the assay used to identify them has been published [Todorovic et al., 1999].

The human hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene is found on the X chromosome (Xq26), and is constitutively expressed. It belongs to a class of housekeeping genes that includes DHFR, adenosine deaminase, and phosphoglycerate kinase, all of which have a similar organization of promoter elements. A report by Subramanian and Chinault [1997] suggested the presence of a replication origin in the HPRT gene region. Further, Sykes et al. [1988] had previously reported the presence of an autonomously replicating site (ARS) in the first intron of the human HPRT gene. Not all sequences exhibiting ARS activity, however, function as origins of replication in their native chromosomal location [DePamphilis, 1996]. Therefore, we analyzed the ARS/MAR region described by Sykes et al. [1988] for origin

Grant sponsor: PHS; Grant numbers: CA84493, ES09112.

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Received 18 October 2001; Accepted 9 January 2002
DOI 10.1002/jcb.10136

activity. A functional origin of DNA replication was located in intron 1 of the HPRT gene, adjacent to the promoter and exon 1. This origin is situated approximately 3.5 kb upstream of the ARS reported by Sykes et al. [1988].

MATERIALS AND METHODS

Cell Culture and Isolation of Short Nascent DNA Fragments

Two sources of diploid human male fibroblasts were used in this study: hTERT-GM01604, telomerase-immortalized fibroblasts from fetal lung [Ouellette et al., 2000] and NHF1, a strain of fibroblasts previously isolated in this laboratory from neonatal foreskin [Boyer et al., 1991]. Cells were cultured in Eagle's minimal essential medium (GIBCO-BRL, Grand Island, NY) supplemented with 2 mM L-glutamine (GIBCO-BRL) and 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT). Logarithmically growing cultures of human fibroblasts were harvested by trypsinization. Cells were washed twice with phosphate buffered saline (PBS) and DNA was prepared as described in Abdurashidova et al. [2000]. Up to 100 μg of DNA was resuspended in 1 ml of TE (10 mM Tris, pH 8.0, 1 mM EDTA), denatured by boiling for 5 min, quickly chilled on ice, and layered on top of a 5–30% neutral sucrose gradient (36 ml total). Gradients were centrifuged at 26,000 rpm for 17 h in a Beckman SW28 rotor, and 1 ml fractions were collected from the top. A gradient containing double stranded DNA size markers was also centrifuged and collected in the same manner. DNA was ethanol precipitated from each fraction of the marker gradient, resuspended in 15 μl of TE, and resolved on a 0.8% agarose gel run at 5.5 V/cm. On the basis of the sedimentation profile of markers (data not shown), fractions containing single stranded DNA fragments ranging from 900 to 1,700 nucleotides were pooled and DNA precipitated with ethanol after the addition of 20 μg of oyster glycogen. The resulting pellet was resuspended in 200 μl of TE and used directly for PCR determinations. Four independent preparations of size fractionated nascent DNA (two from each fibroblast source) were used in this study. In one experiment (1604b), the selected DNA fragments ranged from 900 to 1,500 nt because the fraction containing fragments of approximately 1,700 nt was not included.

Preparation of DNA Used As Standards

Genomic DNA was isolated from hTERT-GM01604 or NHF1 cells and purified for use as reference DNA for the PCR quantification of HPRT sequences in size-fractionated nascent DNA. Before being used as a standard in non-competitive PCR (see below), genomic DNA was repeatedly freeze–thawed to reduce its molecular weight and minimize the presence of secondary structure that might interfere with amplification. For competitive PCR studies, standard DNA was purified from nuclei of confluence-arrested fibroblasts, isolated by organic extractions, precipitated with isopropanol, and resuspended in TE. DNA was sonicated on ice with a Branson model W140D sonifier for 21 s in seven pulses of 3 s, each at 32% power. This treatment resulted in fragments with a size distribution between 500 and 3,000 bp, with a peak at approximately 1,500 bp, as estimated by agarose gel electrophoresis and ethidium bromide staining.

Primer Design and Competitor Construction

Primers were designed using the Prime program in the Wisconsin Package (Genetics Computer Group, Madison, WI, version 8), Prime3 (www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi), or Vector NTI (InforMax Inc., version 6). All HPRT primers were designed using GenBank sequence AC004383 and are listed in Table I. The lamin L5 product corresponds to bases 3984–4193 of GenBank accession number M94363 (forward primer: GGTTCTGCCTCTGAGTTTATTCC; reverse: TGACGAAGAGTCAGCTTGTG). The B13 primer set was the same one reported by Giacca et al. [1994].

Competitive molecules were designed using the PCR MIMIC protocol from Clontech Laboratories (Palo Alto, CA). This entails the construction of a synthetic molecule (competitor) that amplifies with the same primer set as the target molecule, but generates a product of different size. Strehl et al. [1997] used this method to construct PCR competitors for studies of the timing of replication of DNA sequences across an R/G band transition zone on human chromosome 13. Chimeric primers were designed, in which primer sequences specific for the HPRT region (as indicated in Table I) were contiguous to the 5′ end of primers amplifying a region of pUC19 (GenBank accession number M77789).

TABLE I. Primer Sequences and PCR Conditions

Primer set	Primer name	Sequence	Location (nt) in AC004383	Annealing temp (°C)
1	1F	ACCAGTTGGTAAGTCAGTGC	38450–38469	53
	1R	AGATGATACCATTGCACTCC	38675–38694	
2[a]	2F	TAAGGAATTGGCTCACGCA	40012–40030	53
	2R	GATCAAGAAGGCATTCTCCAG	40125–40145	
3	3F	ATCTGGTACTGCAGGATTTG	42090–42109	53
	3R	CTTCCGGTTATATATTCGTCC	42210-42230	
4	4F	ATAAGAAATGGTGTTGCTGG	43623–43642	53
	4R	AGTCAGCAAATGGGAACTAC	43763–43782	
5[a]	5F	CTGATCTGGGTGACTCTAGG	44299–44318	53
	5R	AAGACCTTGCACTACCTGTG	44393–44412	
6	6F	GTTCGGCTTTACGTCAC	45039–45055	49
	6R	GCACATGTCAACCAAAAC	45161–45178	
7[a]	7F	GACCAGGTTTTGCCTTTAG	45363–45381	53
	7R	TCTAGCACATGTGAATTTCG	45517–45536	
8[a]	8F	CGTAATCAGCCTCTGGTATC	45615–45634	53
	8R	AACAGGTAACCAGGTCATTG	45793–45812	
9	9F	TACTAGTTGTGTGGCTGTGG	46053–46072	50
	9R	ATCATACCATTGCTACAGGG	46265–46284	
10	10F	ATTATTGCTAGAGCTGGCTG	47233–47252	53
	10R	TATTGTTGGGTAATCTTGGG	47462–47481	
11	11F	TGTGAATTTAGCACTGTGACC	48110-48130	50
	11R	ACTCCAAGGCAACTCTTCTC	48217–48236	
12	12F	GCCCATGTCCCTAGAATGAG	48678–48697	50
	12R	ACTTGCTTGCCTTCCCTTC	48771–48789	
13	13F	AAATTATCCAAGGAGATGGC	48924–48943	53
	13R	GGCAGAAATTGCTAGTTGG	49013–49031	
14	14F	TTACCACTTCTAGGCCCCTC	50403–50422	50
	14R	GTCCATAACAAGCACCCAAC	50508–50527	
15[a]	15F	GCCTGAGATTGAAACCTACC	51069–51088	49
	15R	CAACACCTTCTGAAAGAACAAC	51196–51217	
16	16F	ACTGCATATCTGGGATGAAC	54061–54080	53
	16R	AGTTACTCACTGCTTCTGCC	54246–54265	

[a]Primer sets that were used for competitive PCR.

Primer set A amplifies a 300-bp sequence (forward: GCGGTGGTTTGTTTGC; reverse: GGTCGTTCGCTCCAAG) and primer set B amplifies a 188-bp sequence (forward: CCGGATCAAGAGCTACCAAC; reverse: AGACACGACTTATCGCCAC) of pUC19. The final competitor size is equal to the size of the pUC19 fragment amplified plus the size of the HPRT specific primers. For each competitor, the pUC19 primer set used was that which generated a competitor closest in size to the specific product that could still be separated on a 2% agarose gel. The chimeric primers were used in a first round of PCR amplification with pUC19 as the template. The specific primers were used in a second round of PCR amplification, in which 1 μl out of 50 μl from the first reaction was used as template. The product from the second round of amplification was purified by agarose gel electrophoresis and quantified in a spectrophotometer. Competitors were serially diluted and quantified more precisely by co-amplification with a fixed amount of genomic DNA sonicated to an average size of 1.5 kb. This DNA is similar in size to the nascent DNA, and is expected to amplify with the same efficiency. Control experiments were performed to identify the conditions under which competitor and target DNA amplification were within the linear range.

PCR

The following reaction conditions were used: 1.25 U Amplitaq Gold (Perkin-Elmer Applied Biosystems, Foster City, CA) with buffer provided by the manufacturer, 200 nM of each primer, 200 μM dNTPs, 2 mM $MgCl_2$, and 0.5–25 ng of DNA in a total reaction volume of 50 μl. PCR was performed using Omn-E PCR thermocyclers (Hybaid Ltd., Ashford, Middlesex, UK). Cycle times were as follows: 95°C for 12 min (one time, to activate the polymerase), 29–36 cycles of 94°C for 30 s, annealing temperature (Table I) for 30 s, and 72°C for 30 s. A negative control without template DNA was included with each set of PCR reactions. PCR products were separated by gel electrophoresis in 2% agarose in TAE (40 mM Tris-Acetate, 1 mM EDTA), and visualized with ethidium bromide. No extraneous bands were generated with any of the primer sets when using either genomic or nascent DNA as template. All PCR reactions

were run in the linear range of amplification. Images of ethidium bromide stained gels were captured and the relative band density analyzed with the AlphaInnotech imaging system and AlphaEase software (San Leandro, CA, version 5.5). Each image was reversed (negative image) to allow for easier viewing and printing. Results were graphed using Microsoft Excel 2000.

As a first approach to the localization of the HPRT replication origin, the sequence amplified by each primer set was quantified non-competitively. A PCR-generated standard curve of genomic DNA was used to determine the relative copy number in the size-selected nascent DNA preparation of specific HPRT sequences. This test was repeated at least three times for each primer set, using the same sample of nascent DNA from hTERT-GM01604 cells (preparation 1604a). The copy number for each DNA region in nascent DNA preparations was calculated from its nanogram equivalent in genomic DNA.

After determining the DNA region most likely to contain the replication origin from its peak abundance in nascent DNA (Fig. 1), five primer sets were chosen to confirm the position of the origin by competitive PCR. The primer sets used for this analysis are marked with an 'a' in Table I. For competitive PCR experiments, increasing amounts of competitor DNA were mixed with a constant amount of nascent DNA or sonicated genomic DNA. Each primer set was tested at least three times using the same nascent DNA preparation (1604a). In parallel, a nascent DNA preparation from NHF1 cells (NHF1a) was analyzed with three of the competitive primer sets (#2, 7, and 15).

RESULTS

The localization of an origin of replication using measurements of sequence abundance in nascent DNA by quantitative PCR relies on the fact that nascent DNA strands grow in length bi-directionally following initiation at

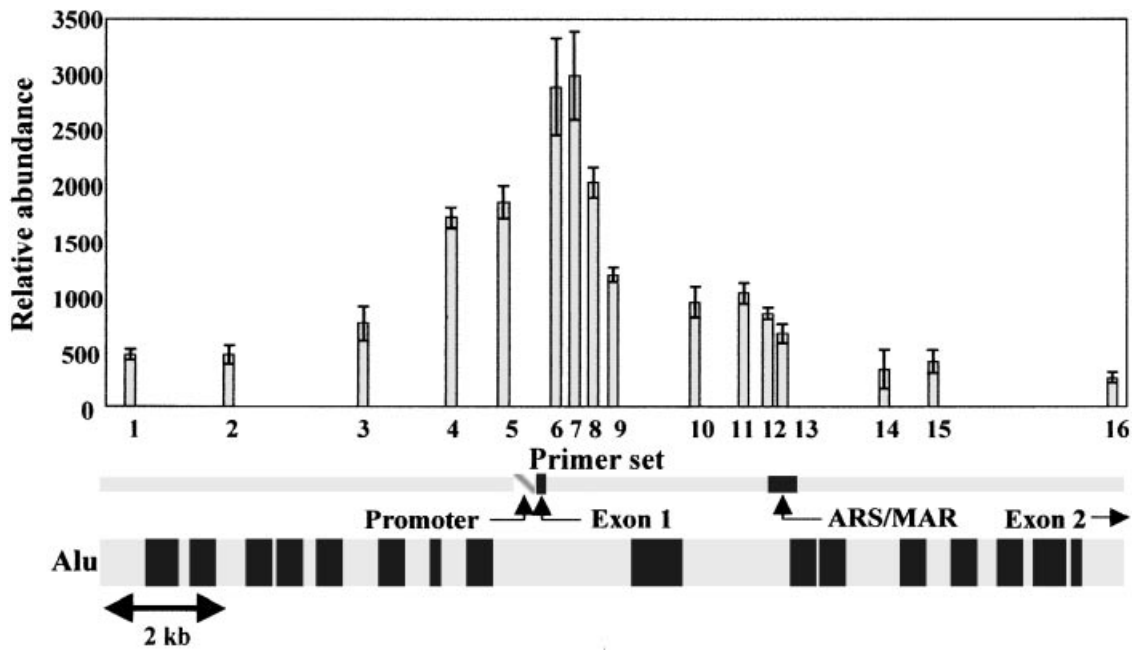

Fig. 1. Non-competitive quantitative PCR mapping of an origin of DNA replication in the human HPRT region. The relative abundance of 16 PCR markers in a preparation of nascent DNA (1604a) is illustrated. The position of each primer set in the chart is aligned with a schematic representation of the HPRT region shown below the chart. The exact location, as well as the nucleotide sequence of each primer is listed in Table I. The positions of the transcriptional promoter and exon 1 of HPRT, as well as the ARS/MAR and alu elements are indicated. The position of alu elements was determined using the repeat finder function in Sequin software (www.ncbi.nlm.nih.gov, version 2.80). The copy number of each PCR marker in the nascent preparation was determined by comparing the amount of product generated from a fixed amount of nascent DNA to a standard curve using genomic DNA as the PCR template. Primer sets were tested at least three times and error bars indicate the standard deviation of the mean.

the origin. Regions that are at or near the replication origin are represented in greater quantities in short nascent fragments than those that are further from the origin. The newly synthesized single stranded DNA used in this study ranged in size approximately from 900 to 1,700 nt. Nascent DNA in this size range is large enough to avoid the inclusion of Okazaki fragments originated from anywhere along the replicon, but it is small enough to be close to the replication origin. Activity of a known origin of replication in the lamin B2 gene was used as a control for the quality of nascent DNA preparations. This origin was shown to be active in fibroblasts, as well as other cell lines [Biamonti et al., 1992; Giacca et al., 1994; Kumar et al., 1996]. Two reference points were chosen for this analysis, one located near the center of the origin (L5 primer set), the other approximately 6 kb downstream at sequences found in low abundance in nascent DNA preparations (B13 primer set). Each of the four preparations of nascent DNA used in this study (1604a, 1604b, NHF1a, and NHF1b) was tested at least three times, using genomic DNA to generate a standard curve so that copy number could be determined. The L5/B13 copy number ratios ranged from 2 to 4 for the preparations of nascent DNA used to characterize the HPRT origin. As an additional control to demonstrate that the DNA isolated by sucrose gradient centrifugation was newly replicated (as opposed to random fragments of genomic DNA), the relative abundance of a sequence in the HPRT gene (primer set 7, Table I) was determined in nascent DNA prepared from an equal number of cells from a logarithmically growing (59% cells in S phase) and a confluent (7% of cells in S phase) culture. An eight-fold enrichment was detected in the relative abundance of HPRT sequences in the nascent DNA preparation from logarithmically growing cells vs. the size-fractionated DNA recovered from the confluent culture (data not shown).

The diagram in Figure 1 shows the copy number of target molecules in nascent DNA preparation 1604a for the 16 primer sets used to analyze the HPRT region (Table I). The positions of the primer sets in the HPRT gene are shown relative to exon 1, the transcriptional promoter, the ARS/MAR [Sykes et al., 1988], and alu elements. PCR primer sets were designed to cover the entire region of interest, while avoiding alu elements. Extremely high GC content also precluded putting primer sets at some sites in the region. The initial PCR analysis was done without competitor in order to establish the general distribution of abundance of specific HPRT sequences in size-fractionated nascent DNA. Although not as precise as quantitative PCR using a competitor, this approach greatly decreases the amount of time needed to complete this first step in the analysis of a novel origin. Initial testing in this region centered on the reported ARS/MAR area (primer sets #11-#13). The representation of these sequences in short nascent DNA was relatively low. Further PCR testing showed a peak of relative abundance around primer sets #6 and #7, which amplify a region located approximately 200 bp from the transcriptional promoter of the HPRT gene.

In order to increase the accuracy of the analysis, competitive PCR was performed. The use of an internal competitor reduces the tube-to-tube variability during PCR and gel loading [for a complete review, see Giacca et al., 1997]. A specific competitor was designed for each of five primer sets chosen for their position relative to the putative origin; one at the abundance peak (#7), and two on each side, in close proximity to the peak (#5 and 8), and further away (#2 and 15) (Fig. 1; Table I). The same preparation of nascent DNA used to generate the data in Figure 1 was used for competitive tests. Figure 2 shows examples of ethidium bromide stained agarose gels with PCR products and graphic analyses of the ratio of competitor to target for the primer sets used. PCR results from parallel reactions with sonicated genomic DNA are not illustrated. Each analysis relied on six data points to correlate the copy number of the competitor in the reaction with the ratio of PCR products from the competitor and the nascent DNA target. A ratio of 1 indicated a copy number of competitor that was equal to the number of target molecules in the nascent DNA preparation. Each primer set was tested at least three times. We noticed that for some primer sets, the expected decrease in the intensity of the target DNA bands (nascent or genomic) was not seen as the competitor concentration was increased. The explanation for this phenomenon is not immediately apparent. However, it was also noticed in some of the PCR results reported by other investigators [Pelizon et al., 1996; Araujo et al., 1999]. Perhaps the range of competitor copies used was not always sufficient

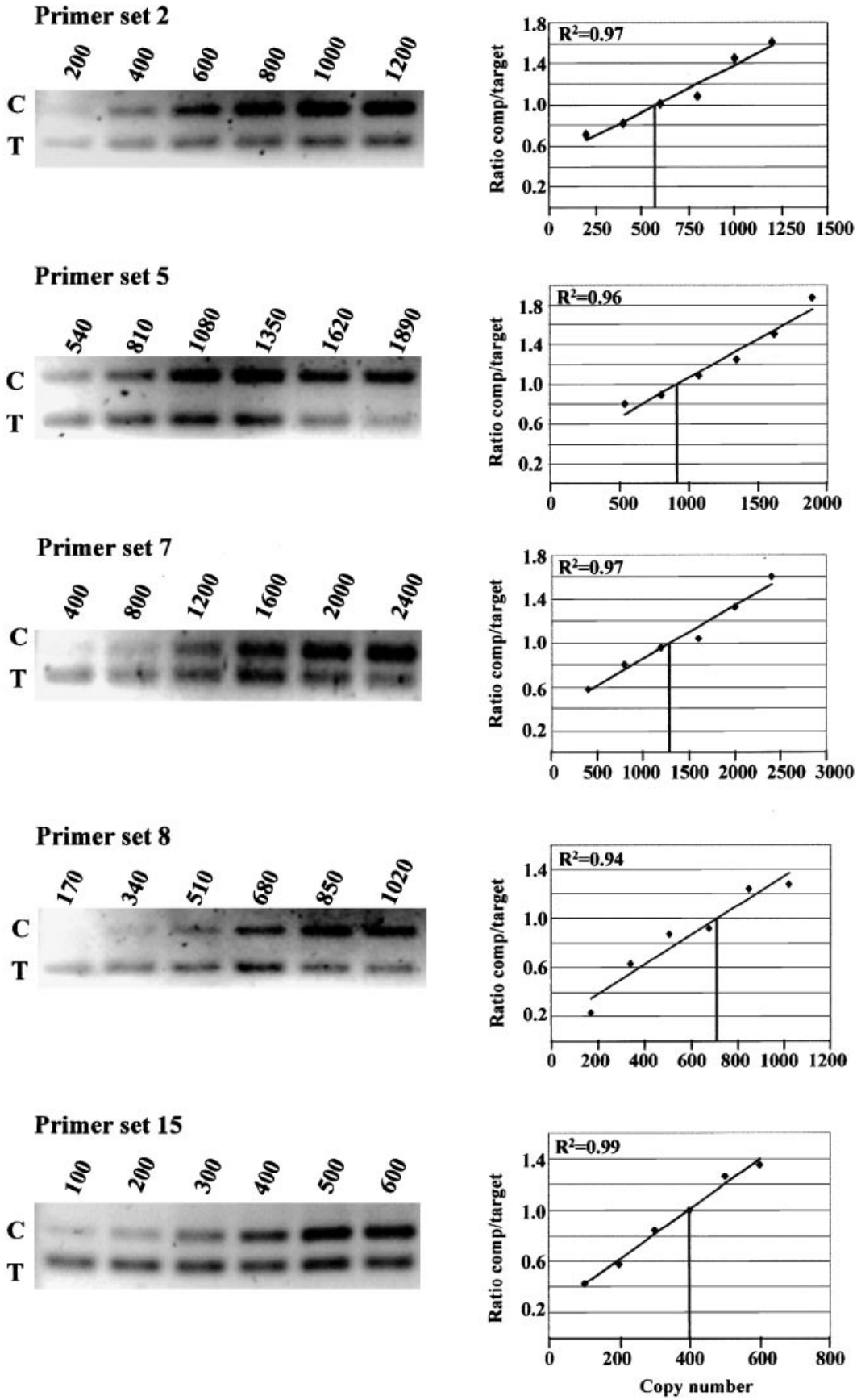
Primer set 2
200
400
600
800
1000
1200
C
T
R²=0.97
Ratio comp/target
1.8
1.4
1.0
0.6
0.2
0
250
500
750
1000
1250
1500
Primer set 5
540
810
1080
1350
1620
1890
C
T
R²=0.96
Ratio comp/target
1.8
1.4
1.0
0.6
0.2
0
500
1000
1500
2000
Primer set 7
400
800
1200
1600
2000
2400
C
T
R²=0.97
Ratio comp/target
1.8
1.4
1.0
0.6
0.2
0
500
1000
1500
2000
2500
3000
Primer set 8
170
340
510
680
850
1020
C
T
R²=0.94
Ratio comp/target
1.4
1.0
0.6
0.2
0
200
400
600
800
1000
1200
Primer set 15
100
200
300
400
500
600
C
T
R²=0.99
Ratio comp/target
1.4
1.0
0.6
0.2
0
200
400
600
800
Copy number

Fig. 2.

to cause a detectable decrease in the density of the template band. Because all PCR reactions were done in the linear range of amplification for both competitor and template DNA, the competitor always functions as an internal standard. Furthermore, we quantified our competitor molecules by co-amplification with sonicated standard. This reaction was always performed in parallel to the co-amplification reactions of competitor and nascent DNA. Since the behavior of the competitor with the sonicated standard mirrored the behavior of the competitor molecule with the nascent DNA, a copy number relative to one standard (sonicated genomic DNA) could be applied to all primer sets tested. This experimental design engenders confidence that the results reported here are not artifactual.

Results for the five competitive primer sets used to analyze the 1604a preparation, as well as the three primer sets used for the NHF1a preparation, are shown in Figure 3. The competitive PCR results were not qualitatively different from those without competitor, although a decrease was noticed in the ratio between the relative abundance at the peak and the tails of the distribution illustrated in Figure 3, compared to that in Figure 1. Nonetheless, the highest copy number was found for sequences amplified by primer set #7 in both quantitative analyses. Two other preparations of nascent DNA from hTERT-GM01604 (1604b), and NHF1 cells (NHF1b), were tested quantitatively, but non-competitively, against the sonicated genomic DNA standard using primer sets #2, 7, and 15, to establish that the peak of abundance was found at the same location in the HPRT gene in different preparations of nascent DNA. The results shown in Figure 4 are consistent with those illustrated in Figures 1 and 3. The differences in the ratios of copy number at the peak and flanking regions in the distributions shown in Figures 1, 3, and 4 might reflect uneven effects on PCR amplification of secondary structure along the HPRT region in the longer genomic DNA standard used to generate the data shown in Figure 1.

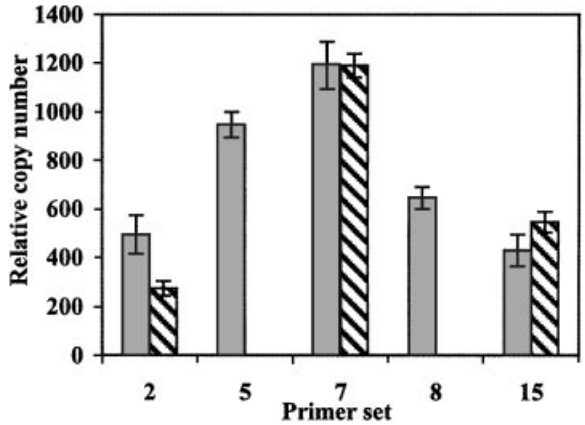

Fig. 3. Summary of competitive PCR experiments. The relative copy number in nascent DNA for each PCR primer set is shown graphically. Results were compiled from experiments as those shown in Figure 2. Data for both the 1604a (solid bars) and NHF1a (striped bars) are shown. Five primer sets were tested for 1604a and three for NHF1a. Each primer set was tested at least three times and error bars indicate the standard deviation of the mean. The copy numbers determined for NHF1a, were normalized to that of 1604a relative to the peak of abundance (primer set #7).

The "copy number" information used in Figures 1, 3, and 4 should not be considered as absolute values, since they only reflect the number of copies within a specific volume of a given preparation of nascent DNA. The actual yield of nascent DNA varies from preparation to preparation due to the number of steps used in its isolation and the fraction of cells that are in S phase. Therefore, the copy number referred to in this article is arbitrary and is not normalized to cell number. It is the distribution of the relative abundance among short intermediates of DNA replication surrounding a defined region of the genome (and not absolute copy number) that indicates the presence of an active

Fig. 2. Competitive PCR analysis. Five primer sets were used for competitive PCR analysis of the 1604a nascent DNA preparation. The sequence for each primer set is listed in Table I. **Left side**: Examples of ethidium bromide stained agarose gels for the primer sets used, illustrating the separation of PCR products from the competitor (C) and the target sequence (T) in the nascent DNA preparations. The number of copies of competitor added to each PCR reaction is shown on the top of each gel. Increasing amounts of competitor were added to a fixed amount of nascent DNA from the same preparation used in the experiments illustrated in Figure 1. **Right side**: Graphs show the ratio of products (competitor to nascent DNA target) plotted against copy number of the competitor. The amount of product was calculated by determining the integrated area of each band from image scans. The R^2 value indicating the degree of linearity of the regression line is shown for each graph. All six data points from each experiment were used in the analyses. When the competitor/target ratio is 1, as indicated by the vertical line on each graph, the number of copies of the target in the nascent DNA preparation is equal to the number of copies of competitor.

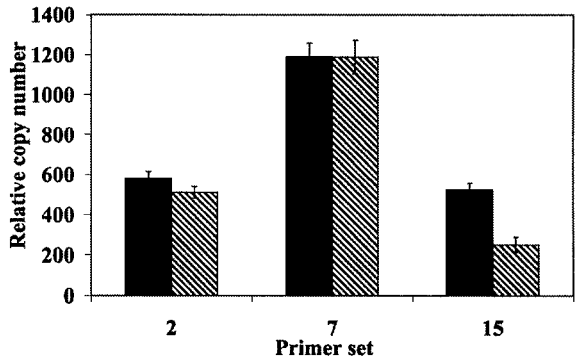

Fig. 4. Non-competitive quantitative PCR results with additional preparations of nascent DNA. In order to demonstrate reproducibility, two additional nascent preparations were tested. Copy number was determined as described in Figure 1, except that the sonicated genomic DNA was used to generate the standard curve. Results are shown graphically. Data for the 1604b (solid bars) and NHF1b (striped bars) nascent preparations are shown. Each primer set was tested at least three times and error bars indicate the standard deviation of the mean. The data from 1604b and NHF1b were normalized to 1604a data (shown in Fig. 3) as described in the legend to Figure 3.

origin of replication within the DNA sequence analyzed.

DISCUSSION

The results presented here indicate that the center of the origin of replication within the HPRT gene, as mapped by the nascent DNA abundance assay, is not at the same position as the ARS/MAR described by Sykes et al. [1988]. In the latter study, plasmids containing fragments of sequence from the HPRT gene region were transfected into yeast cells. Only those clones that originated from the first intron of the HPRT gene were able to replicate autonomously and produced transformants. Further dissection of the intron 1 region indicated that a 580-bp region (48,718–49,298 bp in GenBank sequence AC004383; see Fig. 1) was sufficient to confer ARS activity on the transfected plasmid. This ARS region was also shown to be able to bind very strongly to isolated nuclear matrices, and to contain a region capable of bending. From what little is known about the DNA structure of mammalian replication origins, the ARS/MAR region would appear to be a good candidate for an origin of DNA replication. A diagram of the human HPRT gene showing melting temperatures (T_m) and consensus sequences found in this region was previously reported [Brylawski et al., 2000]. The T_m graph reveals a region of low T_m (high AT content) in the ARS/MAR site that is predicted to be a DNA unwinding element (DUE). Unwinding of the DNA duplex is necessary for replication to begin. The low T_m found at the ARS/MAR site could enhance its ability to unwind easily when inserted into a closed circular plasmid and used in transfection assays. However, the secondary structure of the HPRT ARS region in its chromosomal environment might be quite different from that found in the clones used in the Sykes et al. [1988] study. This would explain why the ARS region does not coincide with the center of the functional origin of replication described here.

The region proximal to the origin mapped in this study is not an area of unusually low melting temperature. It is approximately 200 bp from the transcriptional promoter of the HPRT gene, and is in a region that has a relatively high GC content. Several other mammalian origins of replication were found in close proximity to gene promoters and their CpG dinucleotides. These include the hamster adenine phosphoribosyl transferase (APRT), growth arrest and DNA damage inducible (GADD), and thymidine kinase (TK) genes [Delgado et al., 1998], the human c-myc gene [Tao et al., 2000], and the human Trk A gene [Delgado et al., 1998]. The lamin B2 origin is located at the 3′ end of the gene, but near the promoter of the ppv1 gene [Biamonti et al., 1992].

Recent studies have looked at the relationship between methylation status of CpG regions and origin activity. Some origins are methylated at their CpG regions, such as the DHFR ori-β and ori-RPS14 in CHO cells [Rein et al., 1999] and β-globin in HeLa cells [Araujo et al., 1998]. There is evidence that methylation is important for the function of the hamster DHFR ori-β. Hypomethylation resulted in a loss of origin activity at the DHFR ori-β and ori-β′ sites in hamster cells [Rein et al., 1999]. There appears to be another class of replication origins that remains non-methylated at associated CpG dinucleotides, namely the human c-myc and lamin B2 origins [Araujo et al., 1998; Rein et al., 1999]. This also seems to be the case for the origin mapped in the present study. There is evidence that the CpG dinucleotides near the HPRT promoter on active X chromosomes are not methylated [Hornstra and Yang, 1994]. The heterochromatic, inactive X chromosome in female cells does not transcribe HPRT, and is methylated at the CpG sites associated with the HPRT promoter [Hornstra and Yang, 1994]. Since the present study was done in male

cells, where the only X chromosome present is active, we expect the origin of replication shown here to be associated with CpG dinucleotides that are in the non-methylated state.

In order to understand how a DNA region might function as an origin of replication, some indication of how it functions as a promoter might be useful. The transcriptional promoter for HPRT, like many housekeeping genes, lacks the typical TATAA box found in many promoters, has multiple start sites for transcription, and is GC rich. Although the exact mechanism by which this type of promoter operates is not known, it has been suggested that a stem-loop structure (or cruciform if one considers both strands) is found at the transcriptional start site in genes associated with active CpG rich promoters [Ackerman et al., 1993]. Sequence data indicated that the experimentally determined minimal sequence needed for promoter activity in the mouse adenosine deaminase gene (ADA) could form such a stem-loop structure. When sequence data for the human ADA promoter was assessed in a similar manner, the potential stem-loop structure looked very similar to the stem-loop for the murine ADA promoter, even though the sequence homology between the two was low. The single stranded loop that is part of the structure occurs where the preferred start sites for transcription are found. Further, in this model, methylation at the CpG dinucleotides appears to reduce the formation of stem-loop structures [Murchie and Lilley, 1989], thus leading to inactivation of the gene [Jiralerspong and Patel, 1996]. Stem-loop and cruciform structures might also be important for origin function. It has been proposed that such structures are positioned at or very close to some origins of DNA replication and serve to facilitate duplex unwinding [Pearson et al., 1996]. Although sequence analysis shows a potential stem-loop structure [Kim et al., 1986], there is no direct evidence that such a structure forms in the HPRT promoter region. However, portions of the CpG island region associated with the active HPRT promoter have been shown to be hypersensitive to DNaseI and S_1 nucleases [Wolf and Migeon, 1985], indicating that this region has an open conformation and some single stranded DNA. Nuclease sensitivity has been shown for other CpG island regions, and may reflect a lack of nucleosomes in the area [Tazi and Bird, 1990]. It should be noted that this nuclease sensitivity is not found in the HPRT promoter on the inactive X chromosome [Wolf and Migeon, 1985].

In addition to transcriptional activity, methylation status of the HPRT promoter affects the timing of replication of the gene. There are known differences in the replication timing of genes on the two X chromosomes in female cells: the heterochromatic, inactive one replicates later than the active X. Recent studies have demonstrated that de-methylation at three specific CpG sites is necessary for activation of the HPRT gene in the inactive chromosome [Chen et al., 2001]. De-methylation changes the timing of replication of the inactive HPRT to earlier in the S phase, closer to the replication time of the active allele [Schmidt and Migeon, 1990]. Since the HPRT gene replicates at different times on the two X chromosomes, it is also possible that the two alleles use different replication origins. Both the nuclease sensitivity and the proposed stem-loop structure found at the active promoter are dependent on hypomethylation of CpG islands. Thus, origins of DNA replication located at this type of promoter may be inactive when CpG sites become methylated. At present, there is no definitive answer to this question, but at least one-study reports on the activity of origins of DNA replication on the X chromosome. Rivella et al. [1999] looked at a region of the X chromosome (Xq28) and found 15 potential origins in a 200 kb piece of DNA. These potential origins were identified by hybridization studies performed with amplified nascent DNA from HL60 cells and female lymphocytes. All of the origins were found close to housekeeping and other actively transcribed genes, and are at a much higher density than expected based on an average replicon size of 100 kb. It is likely that not all of these origins fire in every cell. Some may represent alternative sites of replication or may reflect differences in origin usage on the active vs. the inactive X chromosome. Analysis of one potential origin on the inactive X showed that it did not fire during the first two hours of the S phase [Rivella et al., 1999]. It may be that this origin fires later in the S phase (when the inactive X chromosome replicates), or this origin may not be used at all in the inactive X chromosome. Testing of origin activity in hybrid cells carrying only the inactive X chromosome should help in defining whether activity at the HPRT origin of replication is dependent on the methylation status of the promoter.

ACKNOWLEDGMENTS

We are grateful to Dr. Lisa D. McDaniel and Dr. Roger A. Schultz (McDermott Center for Human Growth and Development, University of Texas Southwestern Medical Center) for the gift of the hTERT-GM01604 cell line created with telomerase reagents from Geron Corporation (Menlo Park, CA).

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