Journal of Cellular Biochemistry 78:509–517 (2000)

Construction of a Cosmid Library of DNA Replicated Early in the S Phase of Normal Human Fibroblasts

Bruna P. Brylawski,[1] Stephanie M. Cohen,[1] Jonathan L. Longmire,[2] Norman A. Doggett,[2] Marila Cordeiro-Stone,[1] and David G. Kaufman[1]*

[1]Department of Pathology and Laboratory Medicine, University of North Carolina, Chapel Hill, North Carolina 27599
[2]Life Science Division and Center for Human Genome Studies, Los Alamos National Laboratory, Los Alamos, New Mexico 87545

Abstract We constructed a subgenomic cosmid library of DNA replicated early in the S phase of normal human diploid fibroblasts. Cells were synchronized by release from confluence arrest and incubation in the presence of aphidicolin. Bromodeoxyuridine (BrdUrd) was added to aphidicolin-containing medium to label DNA replicated as cells entered S phase. Nuclear DNA was partially digested with *Sau* 3AI, and hybrid density DNA was separated in CsCl gradients. The purified early-replicating DNA was cloned into sCos1 cosmid vector. Clones were transferred individually into the wells of 96 microtiter plates (9,216 potential clones). Vigorous bacterial growth was detected in 8,742 of those wells. High-density colony hybridization filters (1,536 clones/filter) were prepared from a set of replicas of the original plates. Bacteria remaining in the wells of replica plates were combined, mixed with freezing medium, and stored at −80°C. These pooled stocks were analyzed by polymerase chain reaction to determine the presence of specific sequences in the library. Hybridization of high-density filters was used to identify the clones of interest, which were retrieved from the frozen cultures in the 96-well plates. In testing the library for the presence of 14 known early-replicating genes, we found sequences at or near 5 of them: APRT, β-actin, β-tubulin, c-*myc*, and HPRT. This library is a valuable resource for the isolation and analysis of certain DNA sequences replicated at the beginning of S phase, including potential origins of bidirectional replication. J. Cell. Biochem. 78:509–517, 2000.

Key words: DNA replication; human DNA library; cloning; high-density arrays

The replication of mammalian genomic DNA is a highly coordinated process that involves numerous protein–protein and protein–DNA interactions. This process starts with the activation of a relatively small number of early origins of replication [Tribioli et al., 1987] and proceeds with the firing of a larger subset of replicons, according to an order conserved from cell generation to cell generation. The initiation of replication requires the recognition of DNA *cis*-acting elements, such as unwinding regions, matrix attachment sites, and topoisomerase binding sites by *trans*-acting elements, such as origin recognition proteins, helicases, and polymerases. No consensus sequence for replication initiation in mammalian cells has been reported, and only a few origins of bidirectional replication have been confirmed in human cells [Burhans et al., 1990; Kitsberg et al., 1993; Tasheva and Roufa, 1994; Giacca et al., 1994; Kelly et al., 1995; Aladjem et al., 1995].

Cells in early S phase are most vulnerable to neoplastic transformation [Cordeiro-Stone et al., 1990; Kaufman and Cordeiro-Stone, 1990]. We hypothesized that carcinogen-induced genetic changes that make early replicons more responsive to initiation under conditions in which normal cells are quiescent might constitute an early step in human carcinogenesis. A renewable source of human DNA sequences

Abbreviations used: BrdUrd, 5-bromo-2′-deoxyuridine; TE, 10 mM Tris, 1 mM EDTA; TAE, 40 mM Tris-acetate, 1 mM EDTA; PCR, polymerase chain reaction.

Grant sponsor: United States Public Health Service; Grant number: CA42765.

*Correspondence to: David G. Kaufman, Department of Pathology and Laboratory Medicine, C.B. 7525, University of North Carolina, Chapel Hill, NC 27599-7525. E-mail: uncdgk@med.unc.edu

Received 30 December 1999; Accepted 25 January 2000

that replicate at the beginning of the S phase and should contain functional origins of replication would facilitate the testing of this hypothesis. Several attempts have been made at isolating and cloning DNA replicated early in the S phase from primate [Kaufmann et al., 1985], human [Tribioli et al., 1987; Krysan et al., 1989], or rodent cells [Dimitrova et al., 1993]. These clones were used for studying the DNA sequence interactions with nuclear proteins at the start of DNA replication [Dimitrova et al., 1993], the ability of the cloned sequences to initiate autonomous replication when transfected into mammalian cells [Kaufmann et al., 1985; Krysan et al., 1989], and the unique sequence characteristics of these selected DNA regions [Tribioli et al., 1987]. A common approach in these studies was the synchronization of a cell population to the beginning of the S phase or the G_1/S border, followed by the isolation and cloning of DNA sequences replicated very early in the S phase. Only two of these groups worked with cells of human origin. In one instance [Tribioli et al., 1987], the replicating DNA was purified from transformed cells (HL60), and only a limited number of clones (200) was obtained and analyzed. In the other study [Krysan et al., 1989], putative origin sequences were obtained by transfecting randomly cloned DNA into human cells and isolating, two months later, the clones that were retained inside the mammalian nucleus as autonomously replicating sequences. It was later reported that, in the system used by Krysan et al. [1989], initiation of replication was not due to a specific sequence, but rather it depended on the length of the cloned fragment [Heinzel et al., 1991].

In this laboratory, we have isolated DNA replicated at the beginning and during different windows of the S phase in synchronized rodent [Doggett et al., 1988] and human [Cordeiro-Stone et al., 1990] fibroblasts. Cells were synchronized by a combination of confluence arrest and treatment with the DNA polymerase inhibitor aphidicolin, which allows for the initiation of DNA replication, but dramatically slows replication fork progression [Cordeiro-Stone and Kaufman, 1985; Levenson and Hamlin, 1993] without altering the order of gene replication [Sorscher and Cordeiro-Stone, 1990]. During the incubation in aphidicolin and on removal of the inhibitor, nascent DNA was labeled with bromodeoxyuridine (BrdUrd) at different time intervals during the S phase and was isolated by CsCl gradient centrifugation. In the previous studies, this replicating DNA was used to determine the timing of replication of several genes [Doggett et al., 1988; Cordeiro-Stone et al., 1990]. Several protooncogenes were shown to replicate at the onset of S phase. In this article, we report the successful cloning of early-replicating DNA sequences that incorporated BrdUrd during the incubation in aphidicolin.

METHODS

Cell Culture and Synchronization

Early-replicating DNA was prepared from normal human diploid fibroblasts (NHF1) derived in our laboratory from neonatal foreskin [Boyer et al., 1991]. The cells were grown in Eagle's minimal essential medium (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 2 mM L-glutamine (Life Technologies), and 50 μg/ml gentamicin (Elkins-Sinn, Cherry Hill, NJ). The synchronization protocol included confluence arrest and aphidicolin block, as previously reported [Boyer et al., 1991; Kaufmann and Wilson, 1990; Brylawski et al., 1993], with a few modifications. Briefly, cells were plated at 1.2×10^6 per 100-mm plate, fed on days 3 and 5, and kept undisturbed for 3 days (confluence arrest). They were subsequently replated at 1×10^6 per 100-mm plate. Ten hours later, the culture medium was supplemented with 2 μg/ml aphidicolin, 30 μM BrdUrd, 10 μM 5-bromo-2′-deoxycytidine, and 1 μM trifluorothymidine. The cells were harvested 24 h after replating.

DNA Preparation

Cells were collected by trypsinization, washed twice with phosphate-buffered saline, and nuclei were prepared and lysed according to the protocol recommended by the manufacturer of the genomic DNA purification kit (Qiagen, Chatsworth, CA), except that a 30-min RNase treatment (100 μg/ml at 37°C) was added before the protease digestion. The purified DNA was used for enzymatic digestions without further organic extractions. After isopropanol precipitation, it was resuspended in water and digested with 0.02 or 0.04 U/μg of *Sau* 3AI for 15 min at 37°C in 500 μl aliquots containing 8–10 μg of DNA. The size of the

resulting fragments was determined by gel electrophoresis in 0.4% agarose in 1× TAE at 1 V/cm for 18 h. After the enzyme was inactivated for 10 min at 70°C, the samples were combined and spun in CsCl gradients for the separation of BrdUrd-labeled replicated DNA [Doggett et al., 1988]. Fractions (0.5 ml) were collected from the bottom of the gradients and the absorbance at 260 nm was determined, starting from the fractions collected last (top of the gradient), until the entire peak of the unsubstituted DNA was localized. We compared the gradient position of this peak with those of unsubstituted and hybrid DNA peaks obtained with radioactively labeled DNA samples spun in identical gradients. This approach was used to locate the hybrid-density DNA, avoiding the exposure of the BrdUrd-labeled DNA to UV light and thus preventing photolysis of the DNA to be cloned. Fractions corresponding to the replicated DNA peak were pooled and dialyzed/concentrated in Centricon 3000 spin columns (Amicon, Beverly, MA). All subsequent manipulations of DNA were performed away from direct fluorescent light.

Cloning Vector and Host Cells

Early-replicated DNA obtained from two separate synchronization experiments was cloned into the cosmid vector sCos-1 using methods that have been previously described for the construction of human chromosome-specific libraries [Longmire et al., 1993]. The sCos-1 vector [Evans et al., 1989] contains bacteriophage T3 and T7 promoters flanking a unique *Bam* HI cloning site and *Not* I sites for excision of inserts. It also contains duplicated cos sites, the ColE1 origin of replication, the ampicillin (Amp^r) and kanamycin (Kn^r) resistance genes, and $SV2neo^r$ selection after transfection into mammalian cells. The library was propagated in *Escherichia coli* strain DH5αMCR grown in V-broth (10 g of Bacto tryptone and 5 g of yeast extract per liter, containing 120 mM NaCl, 20 mM KCl, and 0.3 mM $MgSO_4$).

Ligation, Packaging, and Infection

The two DNA preparations were treated separately. DNA was dephosphorylated with calf intestinal phosphatase (Boehringer Mannheim, Indianapolis, IN) following the supplier's recommendations. After heat inactivation, the samples were extracted twice with phenol, extracted once with phenol:chloroform (1:1), and dialyzed three times for 10 min against TE. The resulting DNA (~100 ng) was ligated overnight at 12°C to vector cloning arms using 1 U T4 DNA ligase (New England Biolabs, Beverly, MA). Ligations were packaged using Gigapack Gold extracts (Stratagene, La Jolla, CA) and were infected into host bacteria following the supplier's recommendations.

Growth of the Cosmid Library

After infection and test plating, the primary infection reactions were plated at low density (100 colonies/plate) followed by overnight growth at 37°C. Individual colonies (9,216) were picked, transferred to a well of 96-well microtiter plates (a total of 96 plates) containing growth medium (V-broth with the addition of 30 μg/ml kanamycin), and grown overnight at 37°C (original set). The following day, replica platings of the original set were made for storage and as a source for high-density gridded arrays. An equal volume of freezing medium (2× V-broth containing 40% glycerol) was added to the original plates and to the storage replica sets, which were covered with plastic sealers and stored at −70°C. A Biomek workstation (Beckman Instruments, Fullerton, CA) was used to fill the microtiter plates with growth and freezing media. The material remaining in the wells of the replica plates used to prepare the gridded arrays was combined, mixed with freezing medium, and stored at −80°C. This amplified stock was used in PCR analysis to determine the presence in the library of DNA sequences of interest. Afterwards, the position of positive clones in the high-density grids was identified by hybridization, and single clones were retrieved from the 96-well plates and grown in V-broth containing 30 μg/ml kanamycin. Qiagen plasmid purification kits were used to purify DNA from the amplified pools and from single clones for PCR, fingerprinting by restriction analysis, and Southern hybridization.

High-Density Colony Hybridization Filters

High-density cosmid hybridization filters were constructed using an Adept robotic arm with custom plate-handling and tool-washing modules. The gridding tool is a Los Alamos National Laboratory design, featuring a tool

exchange mechanism and easily replaceable pin tips. Gridding density was 4 identical sets of $(4 \times 4) \times 96$ clones (1,536 clones, equivalent to the contents of 16 plates) onto the surface of a nylon membrane (22 × 22 cm, Hybond-N, Amersham Life Science, Cleveland, OH) layered over an agar plate. This procedure was repeated for all 96 plates in the library. After overnight growth at 37°C, the membranes were processed as described [Nizetic et al., 1991], cut into four sections of 11 × 7.33 cm each, and were stored in plastic bags between two sheets of filter paper (Whatman, Fairfield, NJ) until hybridization. The library is represented by six high-density filters each containing 1,536 clones, for a total of 9,216 clones. Probes labeled with ^{32}P-dCTP (Amersham) were obtained by random priming of isolated PCR fragments using Ready-To-Go kits (Pharmacia Biotech, Piscataway, NJ) following the manufacturer's recommendations. Filters were hybridized overnight at 42°C in a rotating hybridization oven (Robbins Scientific, Sunnyvale, CA). Hybridization buffer contained 1 vol 0.5 M $NaPO_4$, pH 7.4, 1 mM EDTA, 7% sodium dodecyl sulfate (SDS), mixed with an equal volume of formamide and sheared, denatured salmon sperm DNA to yield 100 μg/ml. After hybridization, filters were washed once in 2× SSC, 0.1% SDS for 15 min at 42°C, and twice in 0.1× SSC, 0.1% SDS for 15 min at 55°C. If necessary, an additional wash in 0.1× SSC, 0.5% SDS at 65°C was included. Filters were wrapped in plastic wrap and exposed overnight to Kodak Biomax-MS film at −80° C.

PCR Screening of Amplified Pools and Single Clones

Primers for amplification of gene sequences were designed using the Prime program in the Wisconsin Package (Version 8, September 1994, Genetics Computer Group, Madison, WI). The following reaction conditions were used: 1 U Amplitaq Gold (Perkin-Elmer Applied Biosystems, Foster City, CA) with buffer provided by the manufacturer, 200 nM of each primer, 125 μM dNTPs, 2 mM $MgCl_2$, and 15–100 ng of DNA in a total reaction volume of 50 μl. Template DNA was one of the following: total genomic DNA from NHF1 cells, total cosmid library DNA from pooled stocks, or DNA from individual library clones. PCR was performed using the Omnigene PCR thermocycler (Hybaid, Ashford, Middlesex, UK).

RESULTS AND DISCUSSION

Preparation of Early-Replicating DNA for Cloning

DNA was prepared from two separate batches (100 plates each) of human fibroblasts. As the synchronized cells entered the S phase in the presence of aphidicolin, the newly synthesized DNA was labeled with BrdUrd. The presence of noncycling cells in the population did not affect the quality of the replicating DNA samples obtained, because the DNA containing BrdUrd (and therefore replicated) was recovered from the hybrid-density zone in the isopycnic CsCl gradients. It is unlikely that BrdUrd-substituted DNA deriving from DNA repair activity was present in the hybrid-density region, because repair patches are not big enough to cause a shift in DNA buoyancy away from the peak of unsubstituted DNA [Smith et al., 1981].

Samples from each preparation of BrdUrd-substituted DNA (~500 ng) were used in diagnostic reactions to monitor its partial digestion. We found that a 15-min incubation with 0.02 or 0.04 U of *Sau* 3AI per microgram of DNA provided the best range of fragment length for cloning in sCos-1 [Longmire et al., 1993]. Both conditions were used, and the digested DNA was combined before CsCl gradient centrifugation. Approximately 160 μg of DNA was isolated in each of the two synchronization experiments, restriction-digested, and loaded onto separate CsCl gradients. The amount of replicated DNA recovered after dialysis and concentration was 168 ng and 225 ng, respectively.

Construction of Library and High-Density Gridding

Test infections of the ligation reactions with the preparations of replicated DNA described above gave cloning efficiencies of 7.6×10^5 cfu/μg and 1.6×10^5 cfu/μg DNA. The nonrecombinant background, determined by ligating the same amount of the sCos-1 arms, was 300 cfu, which represents 0.4% and 2% of the clones obtained in the two ligations of replicated DNA. Infection reactions were scaled up to provide an appropriate number of colonies to construct the library. We detected the growth in liquid medium of 8,742 clones of 9,216 that

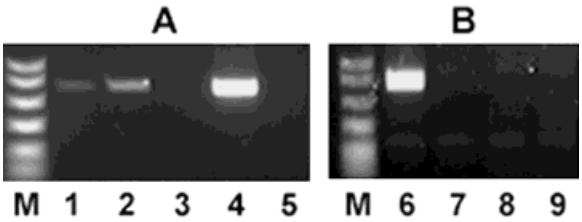

Fig. 1. PCR analysis of the amplified DNA pool of the cosmid library with the β-tubulin primer set. **A:** PCR with amplified stocks as template DNA. M, PCR marker (Promega); **lanes 1 and 2:** 50 and 100 ng of NHF1 genomic DNA, respectively; **lane 3:** 50 ng of amplified stock from plates 1–48; **lane 4:** 50 ng of amplified stock from plates 49–96; **lane 5:** negative control. The β-tubulin gene is present in the library, in the pool from plates 49–96. **B:** PCR analysis with the same primer set using selected clones as template DNA. **Lane 6:** clone 75H01; **lane 7:** clone 76H01; **lane 8:** clone 79H01; **lane 9:** clone 80H01. Clone 75H01 contains β-tubulin.

were transferred individually into the wells of 96 microtiter plates. A working set of plates for gridding and two more sets for storage at −70°C were generated by replica plating of the original 96-well plates. Thirty-six sets of high-density filters were prepared for use in hybridization studies. The average size of the inserts in the cosmid clones was 36 kb, ranging from 23.3 to 43.5 kb, as determined from ethidium bromide–stained gels of *Eco* RI digestion of 32 individual clones.

Screening and Characterization of the Library

We screened the pooled amplified stocks by PCR analysis for the presence of sequences that had been independently demonstrated to be early replicating [Goldman et al., 1984; Holmquist, 1987; Cordeiro-Stone et al., 1990]. Figure 1A illustrates this procedure for the β-tubulin gene. Five of the 14 genes tested were present in the library, as summarized in Table I. When a gene was found in the library DNA amplified stock, the corresponding PCR fragment was gel purified from the control NHF1 DNA amplification reaction (to avoid the possibility of cross-contamination with vector arms), radiolabeled, and hybridized to a set of the high-density arrays to identify and isolate single clones containing the relevant sequence. The high-density filters were initially hybridized to radiolabeled vector arms to verify the growth of the clones on the nylon filters and aid in the determination of the amount of cosmid DNA present in each spot (copy number). Figure 2 illustrates one of the grids from the library hybridized with the vector arms (Fig. 2A), and with the specific probe for β-tubulin (Fig. 2B). Routinely, a few clones located around the site of positive hybridization signal with specific probes were picked and grown, because perfect alignment of the autoradiograms for determining the position of single clones was not always feasible. The DNA from each clone was purified and used for an additional PCR test, as illustrated in Figure 1B. Finally, the purified clone DNA was digested with *Eco* RI, followed by Southern hybridization analysis to confirm the presence of a given gene and further characterize the clone, as illustrated in Figure 3 for the HPRT and β-actin genes. To date, we isolated the clones containing the β-actin, β-tubulin, APRT, and HPRT genes. A clone containing the *ori* region of the *c-myc* gene [Iguchi-Ariga et al., 1988] was also identified and isolated. We tested by PCR the region spanning approximately 8 kb downstream from the origin of c-*myc* in this clone (Table I), and did not obtain a positive signal. This suggested that perhaps we had cloned the area upstream of the c-*myc* gene, for which no data are available from GenBank. If this were the case, the *ori* segment or sequence downstream from it should have been close to one end of the insert. However, when the clone ends were sequenced, none of this sequence was encountered. It is possible that this clone does not contain c-*myc*, but harbors a region of high homology with the c-*myc ori*.

The determination of the level of representation of the DNA source in our library is difficult without a clear definition of what fraction of the total genome should be represented in the earliest replicating DNA isolated under the experimental conditions described above. In our protocol, the cells reaching the S phase would synthesize DNA for at most 12 h (720 min) at a reduced rate in the presence of aphidicolin (2.6% of the DNA synthesis rate observed in the absence of the inhibitor; data not shown). Therefore, the amount of replicated DNA per cell should be equivalent to or less than that observed in 2.6% of 720 min, i.e., 19 min, in the absence of aphidicolin. Because S phase in normal human fibroblasts lasts 8 h (480 min), 19 min of DNA synthesis is equivalent to the replication of at most 4% of the genome. This value is certainly an overestimate, because not

TABLE I. Early Replicating Genes Tested by PCR

Early-replicating gene	Replication time (ref.)	Accession no. of relevant sequence	Sequence location of PCR product (bp as published in Genbank sequence)	PCR results
APRT	First half of S (Goldman, 1984)	U09817	1,194–1,479 (upstream from coding regions)	+
β-Actin	First half of S (Holmquist, 1987)	M10277	Ng et al., 1985	+
β-Tubulin	First half of S (Goldman, 1984)	X00734	8,204–8,849 (3′ of coding region)	+
c-myc	First half of S (Holmquist, 1987), first hour of S (Cordeiro-Stone, 1990)	J00120	13–209 (ori region) 726–843 (ori region) 7,089–7,220 (exon 3) 8,606–8,735	+ − − −
DHFR	First half of S (Goldman, 1984)	X00857	18–140 (exon 4)	−
HPRT	First half of S (Goldman, 1984)	M26434	53,588–53,976 (3′ to exon 9) 17,020–17,319 (intron C)	+ −
K-*ras*	First hour of S (Cordeiro-Stone, 1990)	X02825	33–284 (exon 2)	−
p53	First hour of S (Cordeiro-Stone, unpublished observation)	M13121 J04238	85–668 (exon 11) 100–410 (promoter region)	− −
Lamin B2	First minutes of S (Biamonti et al., 1992)	humlambbb	1,949–2,339 (“last” exon)	−
H-*ras*	First hour of S (Cordeiro-Stone, 1990)	J00277	5,904–6,336 (3′ to gene)	−
c-fos	First hour of S (Cordeiro-Stone, 1990)	K00650	2401–2921 (exon 3, intron 2, part of exon 4)	−
CAD	First half of S (Goldman, 1984)	M38561	1,188–1,669 (intronic)	−
Abl	First 2 hours of S (Cordeiro-Stone, 1990)	U07561	16,702–17,323 (10 kb upstream) 34,964–35,346 (intron 1b)	−
Tyrosine aminotransferase	First half of S (Goldman, 1984)	hstatg5	50–344 (5′ flanking region)	−

all cells proceed through G_1 to S at the same rate, and the average rate of DNA synthesis is lower at the beginning of S phase than later, when most replicons are active. The size of the haploid human genome is 3×10^9 bp, and 4% of that is equal to 1.2×10^8 bp. The number of clones in our library is ~8,750, with average insert length of 36 kb. This results in an estimated total of 3.15×10^8 bp, or an apparent 2× coverage of the early-replicating DNA fraction.

Based on the results from PCR and hybridization experiments, we can assert that our library is enriched with early-replicated elements using a probabilistic analysis. We know that a given gene, for instance, an early-replicating gene such as β-tubulin (6×10^3 bp) is present once in the haploid genome (3×10^9 bp). We will assume that it is completely contained in a 36-kb fragment, the average insert size in our library. The probability of finding a 36-kb fragment of DNA containing β-tubulin by choosing at random from the entire genome (null hypothesis) is 1.2×10^{-5}. Thus, the probability of finding it in 8,742 fragments (or clones), had they been chosen at random from the entire genome, would be $(1.2 \times 10^{-5}) \times 8{,}742$, or 0.105. In other words, of 100 times this test is performed (i.e., independent library construction and hybridization of 8,742 clones chosen at random), the selected gene will be found 10.5 times. Clearly, it would not be possible to validate this number by repeating the

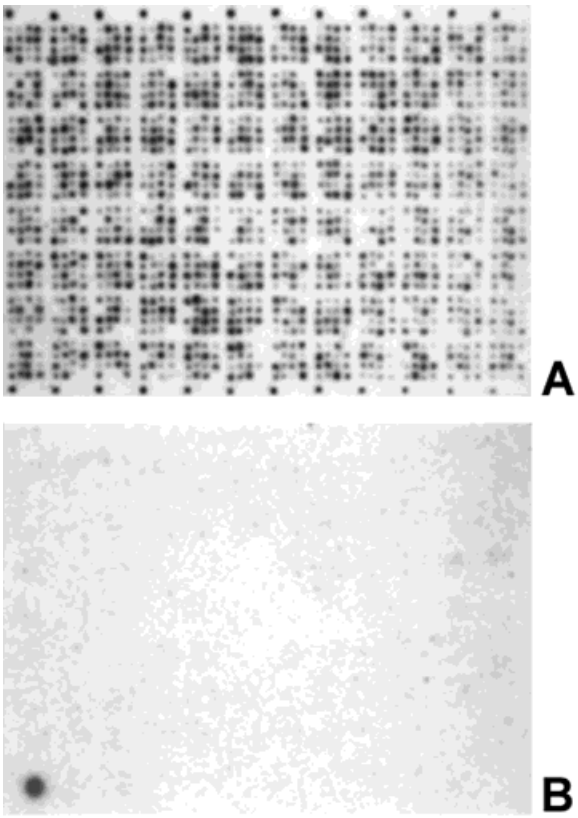

Fig. 2. Hybridization of high-density gridded arrays. **A:** Library filter 5 (containing clones from plates 65–80) hybridized with sCos1. All the spots containing cosmid DNA display signal with intensity proportional to the amount of cosmid (copy number). **B:** Autoradiogram of the same filter hybridized with a radiolabeled PCR fragment specific for β-tubulin.

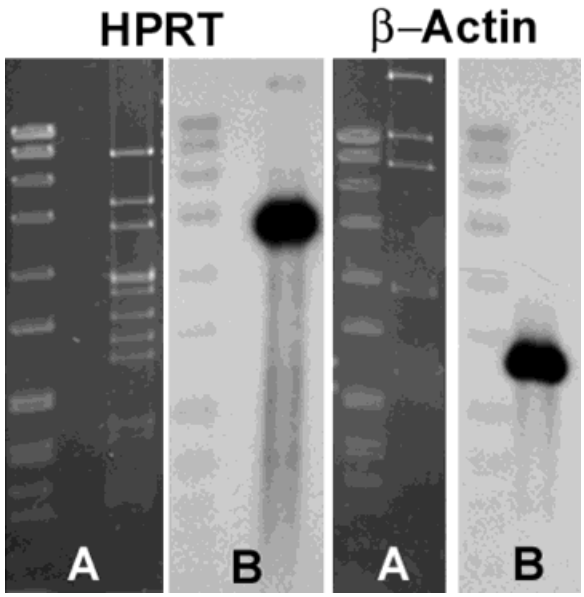

Fig. 3. Southern analysis of two cosmid clones containing HPRT and β-actin. **A:** Ethidium bromide staining of *Eco* RI fragments of individual clones after agarose gel electrophoresis. **B:** Autoradiogram after Southern transfer and hybridization to radiolabeled specific PCR fragment. The first lane in each panel contains DNA size markers (Marker VII, Boehringer Mannheim).

experiment multiple times. Therefore, we calculated that the probability of finding at least five early-replicating genes of 14 that were tested in the same set of 8,742 random genomic clones is 0.011, under the binomial distribution for independent trials. The statistical evidence negates the null hypothesis, indicating that it is quite unlikely that the clones in the library constitute a random collection of nuclear DNA sequences. On the basis of these calculations, and of the procedure we followed for the preparation of the DNA for cloning, we conclude that our library does not represent a random subset of the human genome, but is enriched with early-replicating sequences. The data from the hybridization experiments also seem to suggest that we might have overestimated the coverage of early-replicating DNA in our library. We would expect that at least 50% of the early-replicating genes found in the library would be represented by at least two independent clones, had the coverage been indeed 2× of the source. This observation might be explained by a higher than expected level of contamination of nonsubstituted, unreplicated DNA in the DNA samples used for cloning. This contamination would make the number of clones necessary for full 2× coverage higher than what we chose. Another likely explanation for these findings might rest on the low precision with which the timing of replication of the genes listed in Table I have been determined. Therefore, the genes used to date to test the early-replicating nature of the DNA cloned in our library might not be appropriate for the time interval used to collect the DNA source.

We believe that this library constitutes a valuable resource for the study of the fraction of DNA replicated at the onset of S phase in human fibroblasts. Presumably, these sequences contain origins of early replication and regulatory elements for the start of the S phase. The library can be used for the isolation and study of sequences associated with the nuclear matrix [Brylawski et al., 1993]. We have

shown a positive correlation between the signals derived from hybridization of the library clones with early-replicating DNA and with nuclear matrix–associated DNA [Brylawski et al., 2000]. This library is currently in use for the recovery of sequences found in early-replicating bands in metaphase chromosomes [Cohen et al., 1998], such as band 15q22 and band 1p36.1. Several clones originating from these two bands have been identified in the library, further confirming the early-replicating nature of the cloned sequences.

ACKNOWLEDGMENTS

We thank Erica R. Cobb for her skillful technical contributions, and Professor Thomas H. Brylawski for his help with the statistical analyses. We also thank the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, for making aphidicolin available for this study.

REFERENCES

Aladjem MI, Groudine M, Brody LL, Dieken ES, Fournier RE, Wahl GM, Epner EM. 1995. Participation of the human beta-globin locus control region in initiation of DNA replication. Science 270:815–819.

Boyer JC, Kaufmann WK, Cordeiro-Stone M. 1991. Role of postreplication repair in transformation of human fibroblast to anchorage independence. Cancer Res 51:2960–2964.

Brylawski BP, Tsongalis GJ, Cordeiro-Stone M, May WT, Comeau LD, Kaufman DG. 1993. Association of putative origins of replication with the nuclear matrix in normal human fibroblasts. Cancer Res 53:3865–3868.

Brylawski BP, Cohen SM, Cordeiro-Stone M., Schell MJ, Kaufman DG. 2000. On the relationship of matrix association and DNA replication. Crit Rev Eukar Gene Expr (in press).

Burhans WC, Vassilev LT, Caddle MS, Heintz NH, DePamphilis ML. 1990. Identification of an origin of bidirectional DNA replication in mammalian chromosomes. Cell 62:955–965.

Cohen SM, Cobb ER, Cordeiro-Stone M, Kaufman DG. 1998. Identification of chromosomal bands replicating early in the S phase of normal human fibroblasts. Exp Cell Res 245:321–329.

Cordeiro-Stone M, Kaufman DG. 1985. Kinetics of DNA replication in C3H 10T1/2 cells synchronized by aphidicolin. Biochemistry 24:4815–4822.

Cordeiro-Stone M, Doggett NA, Laundon CH, Kaufman DG. 1990. Timing of proto-oncogene replication and its relationship to transformation sensitivity to chemical carcinogens. In: Paukovits WR, editor. Growth regulation and carcinogenesis. Boca Raton: CRC Press. p 131–142.

Dimitrova D, Vassilev L, Anachkova B, Russev G. 1993. Isolation and cloning of putative mouse DNA replication initiation sites: binding to nuclear protein factors. Nucleic Acid Res 21:5554–5560.

Doggett NA, Cordeiro-Stone M, Chae C-B, Kaufman DG. 1988. Timing of proto-oncogene replication: a possible determinant of S phase sensitivity of C3H 10T1/2 cells to transformation by chemical carcinogens. Mol Carcinog 1:41–49.

Evans GA, Lewis K, Rothenberg BE. 1989. High efficiency vectors for cosmid microcloning and genomic analysis. Gene 79:9–20.

Giacca M, Zentilin L, Norio P, Diviacco S, Dimitrova D, Contreas G, Biamonti G, Perini G, Weighardt F, Riva S, Falaschi A. 1994. Fine mapping of a replication origin of human DNA. Proc Natl Acad Sci USA 91: 7119–7123.

Goldman MA, Holmquist GP, Gray MC, Caston LA, Nag A. 1984. Replication timing of genes and middle repetitive sequences. Science 224:686–692.

Heinzel SS, Krysan PJ, Tran CT, Calos MP. 1991. Autonomous DNA replication in human cells is affected by the size and the source of DNA. Mol Cell Biol 11:2263–2272.

Holmquist GP. Role of replication time in the control of tissue-specific gene expression. 1987. Am J Hum Genet 40:151–173.

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

Kaufman DG, Cordeiro-Stone M. 1990. The roles of cell proliferation and gene replication in neoplastic transformation. In: Paukovits WR, editor. Growth regulation and carcinogenesis. Boca Raton: CRC Press. p 143–151.

Kaufmann G, Zannis-Hadjopoulos M, Martin RG. 1985. Cloning of nascent monkey DNA synthesized early in the cell cycle. Mol Cell Biol 5:721–727.

Kaufmann WK, Wilson SJ. 1990. DNA repair endonuclease activity during synchronous growth of diploid human fibroblasts. Mut Res 236:107–117.

Kelly RE, DeRose ML, Draper BW, Wahl GM. 1995. Identification of an origin of bidirectional replication in the ubiquitously expressed mammalian *CAD* gene. Mol Cell Biol 15:4136–4148.

Kitsberg D, Selig S, Keshet I, Cedar H. 1993. Replication structure of the human β-globin gene domain. Nature 366:588–590.

Krysan PJ, Haase SB, Calos MP. 1989. Isolation of human sequences that replicate autonomously in human cells. Mol Cell Biol 9:1026–1033.

Levenson V, Hamlin JL. 1993. A general protocol for evaluating the specific effects of DNA replication inhibitors. Nucl Acid Res 21:3997–4004.

Longmire JL, Brown NC, Meincke LJ, Campbell ML, Albright KL, Fawcett JJ, Campbell EW, Moyzis RK, Hildebrand CE, Evans A, Deaven LL. 1993. Construction and characterization of partial digest DNA libraries made from flow-sorted human chromosome 16. Genet Anal Tech Appl 10:69–76.

Ng S-Y, Gunning P, Eddy R, Ponte P, Leavitt J, Shows T, Kedes L. 1985. Evolution of the functional human beta-actin gene and its multi-pseudogene family: conservation of noncoding regions and chromosomal dispersions of pseudogenes. Mol Cell Biol 5:2720–2732.

Nizetic D, Zehetner G, Monaco AP, Gellen L, Young BD, Lehrach H. 1991. Construction, arraying, and high-density screening of large insert libraries of human chromosomes X and 21: their potential use as reference libraries. Proc Natl Acad Sci USA 88:3233–3237.

Smith CA, Cooper PK, Hanawalt PC. 1981. Measurement of repair replication by equilibrium density sedimentation. In: Friedberg EC, Hanawalt PC, editors. DNA repair: a laboratory manual of research procedures. New York: Marcel Dekker. p 289–305.

Sorscher DH, Cordeiro-Stone M. Gene replication in the presence of aphidicolin. 1991. Biochemistry 30:1086–1090.

Tasheva ES, Roufa DJ. 1994. A mammalian origin of bidirectional DNA replication within the Chinese hamster *RPS14* locus. Mol Cell Biol 14:5628–5635.

Tribioli C, Biamonti G, Giacca M, Colonna M, Riva S, Falaschi A. 1987. Characterization of human DNA sequences synthesized at the onset of S-phase. Nucl Acid Res 15:10211–10232.