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Isolation of Restriction Fragments Containing Origins of Replication From Complex Genomes

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Abstract

The identification and isolation of origins of replication from mammalian genomes has been a demanding task owing to the great complexity of these genomes. However, two methods have been refined in recent years each of which allows significant enrichment of recently-activated origins of replication from asynchronous cell cultures. One of these involves the selective entrapment of bubble-containing fragments in gelling agarose and their subsequent recovery and isolation by molecular cloning. Libraries prepared by this method from Chinese hamster and human cells have been shown to be extremely pure, and provide a renewable resource of origins that can be used as probes on microarrays or sequenced by high-throughput techniques to localize them within the genomic source. The bubble-trapping method is described here for asynchronous mammalian cells that grow with reasonable doubling times and from which nuclear matrices can be reliably prepared. The method for nuclear matrix preparation and enrichment of replication intermediates is described in an accompanying chapter entitled, "Purifying replication intermediates from mammalian cells by enrichment on the nuclear matrix and BND-cellulose chromatography" (Mesner, Dijkwel, and Hamlin, Chapter ____).

Keywords

Replication intermediates; low melting point agarose; neutral/neutral 2-D gel replicon mapping; recombinant libraries

1. Introduction

In the last 40 years, several different methods have been developed to identify and characterize origins in genomes ranging in complexity from plasmids to the single chromosomes of viruses and bacteria to the multiple linear chromosomes of mammalian cells. Early on, phenotypic rescue methods were developed to identify the *E. coli* origin. These methods relied on a genetic replicator to fuel autonomous replication of a colinear selectable marker (reviewed in Ref. 1). Because virtually all plasmid, viral, bacterial, and budding yeast origins are controlled by and coincide with *bona fide* replicators, this autonomously replicating sequence or ARS assay has been very successful in identifying most of the origins in these simple genomes, and has been particularly powerful when applied to the more complex lower eukaryotic genome of *S. cerevisiae* (2,3). As a consequence, progress in determining the important *cis*- and *trans*-acting elements that are responsible for origin activity in these systems has been remarkable (reviewed in 1,4).

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However, almost any cloned mammalian DNA fragment above a certain size replicates autonomously when transfected into mammalian cultured cells (5,6). In fact, even those of bacterial origin replicate to some degree. Therefore, it has been difficult to identify potential mammalian genetic replicators by this approach. Instead, the 25 or so known higher eukaryotic origins were localized by biochemical approaches that identify the points or regions of initiation in a chromosomal domain of interest. The expectation was that these would coincide with genetic replicators. These methods include: 1) localizing and/or isolating the first fragments to be labeled with radioactive or density-labeled nucleotides at the beginning of S-phase (7–10); 2) determining the direction of fork movement, which should diverge from an origin (11–13); 3) determining the strand bias of either leading (14) or lagging (15) strands in the region of interest, which should switch template strands at an origin; 4) localizing the positions of the smallest nascent strands in the region of interest, which should be approximately centered over origins (16,17); and 5) detecting the unique electrophoretic behavior of those restriction fragments that contain replication bubbles (i.e., origins; 18).

Each of these approaches is technically challenging, particularly in mammalian systems (thus, the small number of origins that has so far been identified after 40 years of effort). Furthermore, most methods focus on chromosomal regions that were previously well-characterized because of interest in their respective genes and for which recombinant clones were already available. As a consequence, the present collection of origins is biased toward those in the neighborhood of transcription units (see ¹⁹ for recent review). What was clearly needed was an unbiased method for isolating origins of replication on a genome-wide scale and positioning them within the genome.

With the availability of genomic tiling arrays that represent large parts or all of the genomes of several complex organisms, as well as affordable high-throughput sequencing, this is now feasible. The required ingredient is a source of nearly-pure origin-centered DNA. In fact, only two of the methods cited above for *localizing* origins actually begin with material partially enriched in origin-containing material (approaches 4 and 5 above). Not surprisingly, therefore, the first whole-genome approaches for localizing origins within complex genomes have relied, wholly or in part, on these well-established isolation methods (20,21). In this article, we will describe a method of origin purification that relies upon the circular nature of restriction fragments that contain replication bubbles: these fragments can be selectively trapped within the matrix of gelling agarose when both components are mixed in the fluid phase. After solidification of the mixture, application of an electric current allows linear fragments and those containing single-forked or X-shaped termination or recombination structures to be eluted (Fig. 1). Bubble-containing fragments from the gelled plug are then recovered and cloned into a suitable vector. The cloning step itself represents a second enrichment step, probably because very long linear contaminants, as well as three- or four-ended single-forked or termination structures, do not appear to be cloned efficiently. We describe the method for preparing comprehensive libraries of start sites from the genomes of cultured mammalian cells, but the process can be scaled down for simpler genomes such as *Drosophila* flies, yeast, and novel bacterial species.

2. Materials (see Note 1)

2.1. Trapping bubble-containing fragments

1. TE: 10 mM Tris HCl, pH 8.0, 1 mM EDTA.
2. TEN: 10 mM Tris HCl, pH 8.0, 10 mM NaCl, 1 mM EDTA.
3. TAE: 40 mM Tris acetate, pH 7.8, 5 mM sodium acetate, 1 mM EDTA.

4. Enriched replication intermediates (~300 µg) purified as described in the companion article (Chapter __, Mesner, Dijkwel, and Hamlin) and dissolved in 3 mL TE. Make sure that the DNA is completely hydrated in TE prior to the trapping procedure.
5. Low melting point agarose (LMP; 2.5 mL of 1% in TE).
6. A 20 × 40 cm horizontal slab gel tank with empty gel tray in place and filled with TAE so that the meniscus is 1 cm above the floor of the gel tray.
7. 1 cc syringes.
8. Single-edged razor blades.

2.2. Recovering trapped fragments from agarose

1. 5× GELase buffer: 200 mM Bis-Tris-HCl, pH 6.0, 200 mM NaCl.
2. GELase (1U/µl; Epicentre Biotechnologies).
3. 10 M ammonium acetate.
4. Absolute ethanol.
5. 5 M NaCl.
6. 70% ethanol.

2.3. Analyzing trapped material for purity

1. 50 µg of enriched replication intermediates (an aliquot of the material in 2.1.4 above) to serve as control.
2. 500 ng of trapped intermediates from Subheading 3.2.4.
3. Materials and equipment for running neutral/neutral 2-D gels as described in Chapter __ (Mesner, Dijkwel, and Hamlin).

2.4. Preparing recombinant origin libraries

1. EcoRI-digested pGEM7 (Promega) or equivalent.
2. 1.5 µg of purified bubble-containing fragments.
3. T4 DNA ligase and 10× buffer (New England Biolabs or equivalent).
4. Electro-competent DH10B bacteria (22).
5. SOB liquid medium: 2% bactotryptone, 0.5% bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄.
6. 2% agar plates in SOB with 50 µg/mL ampicillin.
7. 20% glycerol in SOB (storage medium; 22).
8. Qiagen columns (miniprep and megaprep sizes).

3. Methods

3.1. Trapping bubble-containing fragments (see Notes 2, 3, 4, 6, & 7)

1. 500 µl of replication intermediates (50 µg) and 500 µl 1% LMP (both in TE) are equilibrated separately at 45°C. Mix completely by gentle trituration with a cut-off 1 mL pipettman tip (2 mm diameter bore), and equilibrate at 45°C for one hr (see Notes 2, 3, & 4).

2. Draw up the mixture completely above the narrow neck into a 1 cc syringe without a needle, being careful to avoid bubbling. Place syringe on benchtop and let cool for 2 hr at RT followed by 2 hr at 4°C.
3. Excise the end of the syringe above the neck with a razor blade and use the plunger to gently extrude the agarose directly into the TAE buffer in the gel tank. Position the gels with the long axis perpendicular to direction of the current; equilibrate for 30 min at RT (Fig. 2). Combs can be inserted at this point to somewhat restrain the plugs during placement, but must be removed before electrophoresis. Up to three gels (~150 µg of intermediates) are usually run at once in a 20-cm wide gel box, with the gels aligned as shown in Fig. 2. Make sure that the plugs are completely immersed in the buffer by topping off as necessary.
4. Apply a potential of 0.5 V/cm (~20 mA) for 48 hr at RT, replace the combs, completely replace the TAE in the gel tank, remove combs, and run for an additional 24 hr. This step removes the majority of linear, single-forked, and termination structures from the gel plug (see Fig. 1).

3.2. Recovering trapped fragments from the agarose plug

1. Position combs on either side of the agarose plugs to restrain movement (see Fig. 2), lift gel tray from apparatus allowing buffer to drain, and place tray on benchtop. Cut each plug into 3 pieces, and place each set of 3 pieces into a 1.5 mL microfuge tube. Incubate the three tubes at 70°C for 5 min, and then equilibrate in a 45°C water bath for 20 min.
2. Add 250 µl of 5× GELase buffer to each tube, mix by inverting capped tube, and add 2.5U of GELase. Incubate at 45°C for 60 min, cool to RT, clarify samples by centrifuging at full speed in an Eppendorf microfuge for 2 min at RT. Distribute the supernatant from each tube into three fresh 1.5 mL tubes with a cut-off 1 mL tip, add 110 µl of 10M ammonium acetate and 900 µl absolute ethanol, and incubate overnight at RT (there are now 9 tubes). Note that the nonstandard RT incubation avoids co-precipitation of oligosaccharides in the agarose preparation. The lengthy incubation time facilitates efficient precipitation of the very low concentration of intermediates in the preparation.
3. Centrifuge tubes in Eppendorf microfuge at full speed for 30 min at RT, decant supernatants and blot tubes on paper towel. Air-dry for 5 min, rehydrate each tube in 40 µl of TE at RT for 2 hr, periodically flicking tube to mix. Combine contents of all nine tubes, yielding ~360 µl, rinse each of the three tubes with 40 µl TE, and combine rinses with samples to yield ~480 µl total. Add 25 µl of 5M NaCl and 1 mL of absolute ethanol. Mix by gentle inversion and incubate for at least 60 min at RT. Centrifuge at full speed for 30 min at RT and decant supernatant. Wash pellet with 1 mL of 70% RT ethanol, centrifuge at RT for 15 min, decant supernatant, air dry, and rehydrate in 30 µl of TE for at least 24 hr at 4°C.
4. Measure yield of trapped fragments in a 1 mL aliquot with Hoescht dye and a fluorocolorimeter (23) or a similarly sensitive assay. Note that the yields for successful purifications are normally in the range of 1% of the starting material. That would be 1.5 µg from 150 µg of total replication intermediates in this example for each set of three agarose plugs (50 µg per plug). In practice, we double this amount and trap 250–300 µg of replication intermediates, yielding 2.5–3.0 µg of trapped material. If the yield is much higher than this, it is likely that the trapped material also contains significant amounts of contaminants (i.e., single-forked fragments, recombination intermediates, and long linear fragments).

3.3. Analyzing trapped material (see Note 6)

1. Analyze 500 ng of purified trapped material in 100 μ l from 3.2.4 above, as well as 5 μ g of the starting purified replication intermediates from the same cell line, on a standard neutral/neutral 2-D gel as described in Chapter __ (Mesner, Dijkwel, and Hamlin; also see Note 2 below).
2. A successful trapping of bubble-containing fragments in the starting preparation shown in Fig. 3B should look very similar on by 2-D gel analysis to the pattern shown in Fig. 3C, where >95% of the single fork arc has been removed and only a small *1n* spot remains. Note that when the same blot was re-probed with a non-origin fragment from the body of the DHFR gene, this *1n* spot cannot be detected in a successful trapping (Fig. 3D&E), arguing that the *1n* spot in Fig. 3C represents the linear template of small bubbles that were destabilized during isolation from the agarose plug. This is supported by the observation that the small end of the bubble arc shown in Fig. 3B is clearly depleted in the trapped material shown in Fig. 3C. Therefore, the vast majority of material that enters a 2-D gel arose from bubble-containing fragments. Importantly, however, analysis of the first-dimension of these gels shows that a significant portion of the material trapped in the agarose plug is >50 kb, and undoubtedly represents very long linear fragments and/or large forked structures (including bubbles). Apparently, these large unwanted structures do not clone efficiently. Therefore, the cloning operation itself represents an essential purification step. It is also conceivable that even small tri- and tetra-ended structures are ligated into non-circular structures, which are then not propagated in the bacterial host.

3.4. Preparing recombinant origin libraries

1. Of the 2.5–3.0 μ g of trapped intermediates recovered from 5–6 plugs in Subheading 3.2.4 above, 1.5 μ g of purified, trapped fragments have proved to be sufficient starting material for preparing a comprehensive mammalian origin library (e.g., $\sim 10^6$ independent clones). This assumes that the cloning steps themselves are reasonably efficient. In practice, to maximize the cloning operations, small test ligations are performed initially, followed by transformation into the DH10B bacterial strain. Given the expense of preparing the trapped material in the first place (particularly from mammalian cells), we suggest a dry run that tests the efficacy of the reagents and the bacteria, using 50 ng of the enriched replication intermediates eluted with caffeine from BND-cellulose as test material (see Chapter __, Mesner, Dijkwel, and Hamlin). Therefore, 50 ng of this test DNA, 25 ng of vector (pGEM7 in this example), and 120 U ligase (New England Biolabs) are incubated for 4 hr at 16°C in a volume of 5 μ l. One μ l of ligation mixture is then electroporated into 4×10^9 DH10B cells in 40 μ l of 10% glycerol (BioRad Genepulser II, 0.1 cm cuvette; 1.3 kV/200 Ohms/25 μ F with a decay constant of ~ 4.5 msec). This procedure should yield $3\text{--}4 \times 10^4$ clones per ligation reaction >95% of which contain inserts. When this benchmark is achieved, the procedure would then be repeated with 50 ng of the *trapped* material as the source, to ensure that it yields similar numbers of colonies. Increasing the amount of trapped material or changing other parameters might be required to generate $3\text{--}4 \times 10^4$ clones.
2. Once ligation and transformation have been optimized, the procedure is scaled up to 1.5 μ g of trapped material, 0.75 μ g of pGEM7, and 3600 U ligase (NEB) in a volume of 150 μ l (or the ratios established in Step 3.4.1 above). The DNA in the resulting ligation mixture is precipitated by the addition of NaCl to 250 mM and two volumes of absolute ethanol. After mixing and incubating for 60 min at RT, the DNA is pelleted at full speed in an Eppendorf benchtop microfuge at RT for 30 min. The tube is drained, and the pellet is washed with 500 μ l RT 70% ethanol and recentrifuged. The

supernatant is decanted, and the pellet is air-dried and resuspended in 40 μ l 0.5 $\times$ TE. In 2.5 μ l aliquots, the ligated material is electroporated into 4×10^9 DH10B cells in a total volume of 40 μ l as described above. Following electroporation, each of the 16 cell/DNA mixtures is added to 1 mL of SOB medium without ampicillin and allowed to recover for 60 min with shaking (225 RPM) at 37°C. Each mixture is divided and spread among five 10-cm plates (~80 plates total). Thus, each plate should yield $1-1.5\times 10^4$ colonies per plate (~ $0.8-1.2\times 10^6$ colonies total). Plates are then incubated at 37°C for 8–10 hr or until colonies are first visible by eye to avoid overgrowth. Five mL of 20% glycerol in SOB are added to each plate, colonies are scraped with a policeman, and triturated with a 10 mL pipette. All of the scraped material is pooled, concentrated by centrifugation at $5,000\times g$ for 10 min at 4°C, resuspended in 30 mL SOB-glycerol, and stored in 1 mL aliquots at both –70°C and in liquid nitrogen.

3.5. Assessing quality of libraries (see Note 5)

1. Since many (if not most) mammalian origins appear to correspond to zones of inefficient sites scattered throughout broad zones, a comprehensive library of all the possible start sites could require many-fold more than the ~50,000 origins predicted from early fiber autoradiographic studies (26). Therefore, we have strived to recover at least 10^6 independent clones in order to approach saturation. When this number of clones is obtained as described in Part 3.4 above, the libraries are then characterized by: i) determining the number of clones with inserts as well as the insert sizes, and ii) the percentage of inserts that actually correspond to genomic initiation sites.
2. In our laboratory, we routinely utilize neutral/neutral 2-D gels to characterize replication origins in mammalian genomes. This also would be the method of choice for libraries from the simpler genomes of bacteria, yeast, and flies. In either case, appropriate non-repetitive PCR primers would have to be elaborated to synthesize suitable unique probes for hybridization to the 2-D gel transfers. However, most investigators working on mammalian systems utilize the small nascent strand abundance assay for localizing start sites. This approach requires that several primer pairs be elaborated for each clone, since an isolated fixed origin might reside anywhere within the cloned origin fragment. In practice, the trapping method probably only recovers those fragments in which the replication bubble was somewhere within the central 65–75% of the fragment, since start sites near the end are rapidly converted to single-forked structures (see Note 5). The methods for generating PCR primers below are applicable to both 2-D gel nascent strand abundance analysis.
3. Select ~100 clones from the library, prepare minipreps by standard methods (e.g., the protocol supplied with the Qiagen columns, and digest the recovered DNA with the restriction enzyme used to prepare the original replication intermediates. Separate on a 0.6% agarose gel to determine the percentage of clones with inserts and the distribution of insert sizes.
4. Select ~30 clones whose inserts are 3–15 kb in length, and sequence ~500 bp from the fragment end (s). For many mammalian genomes (e.g., human, murine, rat), this information can be utilized to obtain the entire sequence of the fragment. Select those clones containing ≥ 1 kb of unique sequence that would represent a suitable non-repetitive probe for 2-D gels or non-repetitive primer pairs for the nascent strand abundance assay.
5. Amplify the unique sequences determined above by PCR, using ~100 pg in 50 μ l reactions. Isolate the PCR products, label with ^{32}P -dCTP by random priming, and use to probe 1-D Southern blots of 10 μ g of a genomic restriction digest to ascertain that the probes are unique. If these probes still give background hybridization and you are

confident of the *in silico* analysis, it is helpful to subclone the probe fragment and/or to amplify with an internal primer pair.

6. When suitable probes are prepared for 15–20 independent inserts, utilize them to probe replicate blots of standard 2-D gel separations of replication intermediates isolated from synchronized cells in early S-phase (see Chapter __, Mesner, Dijkwel, and Hamlin). Alternatively, the sequence information can be used to prepare PCR primers to query the potential origin regions in the central part of the fragments by the small nascent strand abundance assay (Chapter __, __).

4.0 Notes

1. All buffers are freshly-prepared, and electrophoresis tanks must be scrupulously clean. For the cloning operations, all media are autoclaved.
2. It is truly important to remember that the entities you are attempting to purify (replication intermediates) are fragile branched structures that will be easily sheared and/or destroyed by branch migration. Therefore, handle with care and store DNA samples at 4°C when not manipulating during the steps described above.
3. We emphasize that for the trapping procedure to be efficacious, it is critically important to make sure that the agarose-to-DNA ratios be within the guidelines stated above and that all components prior to trapping and during mixing come to a homogenous state – be it temperature or uniformity of solutions. Pre-equilibration of the LMP agarose/sample mixture before delivery to the well of the agarose gel and cooling is *critical* for maximum retention of bubbles and minimal retention of contaminating linears, single-forked and X-shaped structures.
4. The method we have described here begins with replication intermediates isolated by enrichment on the nuclear matrix and BND-cellulose chromatography (see Mesner, Dijkwel, and Hamlin, Chapter __). When the method is applied to simpler eukaryotic or prokaryotic genomes, it is important to avoid methods of purification that employ phenol and/or phenol/chloroform extraction, since there is significant loss of replication intermediates during these procedures.
5. The bubble trapping method begins with a restriction digest of genomic DNA, which necessarily positions any replication bubble arbitrarily within the fragment and, if the fragment is large, also limits the resolution of origin localization. In our experience, most origins appear to correspond to zones, so that resolution does not play a large part in localization. However, there may be more fixed origins than have been discovered so far (such as lamin B2; 27). In the latter case, the choice of restriction enzyme could accidentally digest the DNA in such a way as to place the origin near the end of the fragment, in which case it would quickly be converted to a single fork and would not be trapped. Therefore, for true saturation, it would be necessary to utilize two or three additional restriction enzymes to generate independent libraries. This would greatly increase both the coverage and the resolution of origin mapping.
6. Note that the inefficiency of most, if not all, mammalian origins precludes the possibility of detecting active single-copy origins on 2-D gels in preparations of replication intermediates isolated from asynchronous cells. Therefore, although it is possible to isolate libraries from asynchronous cells, determining whether they actually represent true origins requires that the inserts be queried on synchronized cells. In our experience, most of the origins in mammalian cells fire in early S-phase, which would then be the time at which cells should be sampled for 2-D gels. A positive rate of >90% would represent an almost pure library of origins, but most origins that fire later in S-phase will not be represented. When the libraries are prepared from

synchronized cells in early S-phase, the purity can, in fact, approach 100% (20). This same limitation would not apply to simpler organisms in which the origins appear to be more active, more circumscribed, and more closely-spaced. This increase in signal-to-noise ratio then allows the origins identified in an asynchronous library to be tested on 2-D gel separations of genomic DNA isolated from asynchronous cells.

7. Recapitulation of starting cell numbers, manipulations, and resulting libraries:

- A. For libraries containing $\sim 1 \times 10^6$ independent clones from an asynchronous culture of mammalian cells with a doubling time of 18–22 hr, $\sim 5 \times 10^9$ cells will be required (e.g., 160 15-cm plates for HeLa cells at $\sim 3 \times 10^7$ /plate. For longer doubling times, proportionately more cells are needed. This is consistent with the observation that S-phase in different cell lines appears to be similar (8–10 hr) regardless of the overall cell cycle time (unpublished observations). Clearly, for yeast, which contain ~ 300 hundred origins that are usually fixed, only several thousand clones would be required to achieve saturation, and proportionately fewer cells. Our discussion below will focus on mammalian cells, for which ARS assays largely have not been successful in identifying replicators.
- B. 5×10^9 mammalian cells should yield a total of ~ 300 μ g of replication intermediates (60 μ g of intermediates in the caffeine wash of the BND columns for each of the 5 batches of 10^9 HeLa cells; see Chapter __, Mesner, Dijkwel, and Hamlin).
- C. Approximately 5 μ g of the replication intermediates are used for 2-D gel analysis, using the multi-copy rDNA locus as control (see Mesner, Dijkwel, and Hamlin, Chapter __).
- D. Five 50 μ g batches of replication intermediates are trapped individually in five 1 mL agarose plugs as described above (Part 3.1).
- E. Each plug is cut into 3 pieces and treated with GELase in a single tube; the resulting supernatant from each plug is eventually precipitated in 3 separate tubes.
- F. After purification from the plugs, the trapped DNA is pooled to yield ~ 2.5 μ g in 50 μ l TE, which is set aside for library construction.
- G. Approximately 500 ng of this is analyzed on a 2-D gel (see Fig. 3).

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References

- Kornberg, A.; Baker, TA. DNA Replication. 1 edn.. New York: W.H. Freeman; 1992.
- Chan CS, Tye BK. Autonomously replicating sequences in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA 1980;77:6329–6333. [PubMed: 7005897]
- Stinchcomb DT, Thomas M, Kelly J, Selker E, Davis RW. Eukaryotic DNA segments capable of autonomous replication in yeast. Proc. Natl. Acad. Sci. USA 1980;77:4559–4563. [PubMed: 6449009]
- Machida YJ, Hamlin JL, Dutta A. Right place, right time, and only once: replication initiation in metazoans. Cell 2005;123:13–24. [PubMed: 16213209]
- Krysan PJ, Haase SB, Calos MP. Isolation of human sequences that replicate autonomously in human cells. Mol. Cell. Biol 1989;9:1026–1033. [PubMed: 2542763]

6. Haase SB, Calos MP. Replication control of autonomously replicating human sequences. *Nucl. Acids Res* 1991;19:5053–5058. [PubMed: 1656387]
7. Zannis-Hadjopoulos M, Persico M, Martin RG. The remarkable instability of replication loops provides a general method for the isolation of origins of DNA replication. *Cell* 1981;27:155–163. [PubMed: 6276021]
8. Heintz NH, Hamlin JL. An amplified chromosomal sequence that includes the gene for dihydrofolate reductase initiates replication within specific restriction fragments. *Proc. Natl. Acad. Sci. USA* 1982;79:4083–4087. [PubMed: 6955792]
9. Kelly RE, DeRose ML, Draper BW, Wahl GM. Identification of an origin of bidirectional DNA replication in the ubiquitously expressed mammalian CAD gene. *Mol. Cell. Biol* 1995;15:4136–4148. [PubMed: 7623808]
10. Tribioli C, Biamonti G, Giacca M, Colonna M, Riva S, Falaschi A. Characterization of human DNA sequences synthesized at the onset of S-phase. *Nucleic Acids Res* 1987;15:10211–10232. [PubMed: 2827117]
11. James CD, Leffak M. Polarity of DNA replication through the avian alpha-globin locus. *Mol. Cell. Biol* 1986;6:976–984. [PubMed: 3785167]
12. Nawotka KA, Huberman JA. Two-dimensional gel electrophoretic method for mapping DNA replicons. *Mol. Cell. Biol* 1988;1408–1413. [PubMed: 2837639]
13. Muller M, Lucchini R, Sogo JM. Replication of yeast rDNA initiates downstream of transcriptionally active genes. *Mol. Cell* 2000;5:767–777. [PubMed: 10882113]
14. Handeli S, Klar A, Meuth M, Cedar H. Mapping replication units in animal cells. *Cell* 1989;57:909–920. [PubMed: 2544294]
15. Burhans WC, Vassilev LT, Caddle MS, Heintz NH, DePamphilis ML. Identification of an origin of bidirectional DNA replication in mammalian chromosomes. *Cell* 1990;62:955–965. [PubMed: 2393905]
16. Vassilev LT, Johnson EM. Mapping initiation sites of DNA replication in vivo using polymerase chain reaction amplification of nascent strand segments. *Nucl. Acids Res* 1989;17:7693–7705. [PubMed: 2552413]
17. Pelizon C, Diviacco S, Falaschi A, Giacca M. High-resolution mapping of the origin of DNA replication in the hamster dihydrofolate reductase gene domain by competitive PCR. *Mol. Cell. Biol* 1996;16:5358–5364. [PubMed: 8816447]
18. Brewer BJ, Fangman WL. The localization of replication origins on ARS plasmids in *S. cerevisiae*. *Cell* 1987;51:463–471. [PubMed: 2822257]
19. Aladjem, MI.; Falaschi, A.; Kowalski, D. Eukaryotic DNA Replication Origins. DePamphilis, MEd, editor. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2006. p. 31-62.
20. Mesner LD, Crawford EL, Hamlin JL. Isolating apparently pure libraries of replication origins from complex genomes. *Mol. Cell* 2006;21:719–726. [PubMed: 16507369]
21. Todorovic V, Giadrossi S, Pelizon C, Mendoza-Maldonado R, Masai H, Giacca M. Human origins of DNA replication selected from a library of nascent DNA. *Mol. Cell* 2005;19:567–575. [PubMed: 16109380]
22. Hanahan D, Jessee J, Bloom FR. Plasmid transformation of *Escherichia coli* and other bacteria. *Methods Enzymol* 1991;204:63–113. [PubMed: 1943786]
23. Labarca C, Paigen K. A simple, rapid, and sensitive DNA assay procedure. *Anal. Biochem* 1980;102:344–352. [PubMed: 6158890]
24. Dijkwel PA, Vaughn JP, Hamlin JL. Mapping of 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* 1991;11:3850–3859. [PubMed: 2072896]
25. Little RD, Platt TH, Schildkraut CL. Initiation and termination of DNA replication in human rRNA genes. *Mol. Cell. Biol* 1993;13:6600–6613. [PubMed: 8413256]
26. Huberman JA, Riggs AD. On the mechanism of DNA replication in mammalian chromosomes. *J. Mol. Biol* 1968;32:327–341. [PubMed: 5689363]
27. Abdurashidova G, Deganuto M, Klima R, Riva S, Biamonti G, Giacca M, Falaschi A. Start sites of bidirectional DNA synthesis at the human lamin B2 origin. *Science* 2000;287:2023–2026. [PubMed: 10720330]

28. Dean WW, Dancis BM, Thomas CA Jr. The trapping of circular DNA in agarose gels. *Anal. Biochem* 1973;56:417–427. [PubMed: 4765635]
29. Vaughn JP, Dijkwel PA, Hamlin JL. Replication initiates in a broad zone in the amplified CHO dihydrofolate reductase domain. *Cell* 1990;61:1075–1087. [PubMed: 2350784]

Figure 1. Principle of the bubble trapping protocol

The cartoon is modeled after that of Thomas and coworkers (28), who showed that circular plasmid DNAs can be selectively purified from a mixture of circular and linear species in gelling agarose. Here, we show the four different forms of restriction fragments present in a digest of DNA from replicating cells (i.e., bubble-containing, singleforked, X-shaped, and linear species). Only the first of these becomes “concatenated” with the gelled agarose matrix, and therefore cannot be removed by electrophoresis.

Figure 2. Procedure for preparing agarose trapping plugs and electroelution of contaminants
 Panel A. The molten mixture of replication intermediates and agarose is drawn up into a 1 mL syringe somewhat above the small neck and is allowed to solidify (see text). Panel B. This cartoon looks down on a standard flat bed gel box with the gel tray and two combs in place. The gel box contains electrophoresis buffer filled to a depth of ~1 cm above the floor of the gel tray (i.e., enough to completely submerge gels). Three agarose plugs containing replication intermediates are shown in the correct position, which were gently extruded from their syringes directly into the buffer in the gel box with the aid of the plunger, as shown in Panel A. Combs can be inserted prior to electrophoresis to aid positioning of the plugs, but must be removed during electrophoresis. They are repositioned afterwards to aid safe handling of the tray and plugs for further processing (see text). White arrow to the right of gel box indicates that the current is applied in one direction only for the duration of the run.

Figure 3. Analyzing trapped materials on 2-D neutral/neutral replicon mapping gels

2-D gels separations of starting replication intermediates and trapped materials were performed as described in Chapter __ (Brewer). Panel A. Principle of the neutral/neutral 2-D gel method, showing the characteristic migration patterns of linear fragments (curve *a*), single forked fragments (curve *b*), and a centered bubble-containing fragments (curve *c*). Panels B&C. 2-D gel patterns of CHO replication intermediates isolated in very early S-phase before (B) and after (C) trapping; transfers of the DNA were hybridized with a probe specific for the *ori-β* locus in the DHFR initiation zone (29). The characteristic composite pattern shown in Panel B indicates that initiation can occur at several sites within this fragment (including its center), resulting in a complete bubble arc; however, the fragment is usually replicated passively from forks emanating from other sites within the initiation zone (resulting in a prominent and complete single fork arc). Panel C shows that >95% of the trapped material corresponds to replication bubbles. Note the loss of some of the material from the smaller end of the bubble arc and the appearance of a small *1n* spot. Panels D&E. The same transfers shown in Panels B&C were stripped and rehybridized with a probe for the DHFR gene, which does not serve as a template for initiation of replication. The absence of any signal at the *1n* position in the trapped material shows that the *1n* spot seen in Panel C must have arisen from destabilized bubbles, and does not represent contaminating linear fragments.