Critical Reviews in Biochemistry and Molecular Biology, 27(6):445–472 (1992)

Guide to Identification of Origins of DNA Replication in Eukaryotic Cell Chromosomes

Lyubomir T. Vassilev and Melvin L. DePamphilis

Department of Cell and Developmental Biology, Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110

Referee: Dr. Ron A. Laskey, Dept. of Zoology, CRC Molecular Embryology Group, Univ. of Cambridge, Downing Street, Cambridge, CB2 3EJ, United Kingdom

ABSTRACT: Several experimental approaches for identification of origins of DNA replication have been developed recently that allow, for the first time, unique initiation sites in mammalian chromosomes to be mapped at single-copy loci. A brief description of the rationale, advantages, and limitations has been provided for each approach, as well as information that can help the reader choose the method(s) most suitable for a particular system. The various methods are divided into three groups: (1) analysis of nascent DNA strands, (2) analysis of DNA structures, and (3) analysis of origin activity (i.e., ability to support autonomous replication). It is hoped that this information will serve as a practical guide for identifying new origins of replication.

KEY WORDS: eukaryotes, DNA, replication origins.

I. INTRODUCTION

In contrast to bacterial and viral chromosomes that replicate as a single replicon,[44] eukaryotic chromosomes duplicate their DNA by means of numerous replication units. The replication unit, first described by Huberman and Riggs,[41] is an independent unit functionally equivalent to the bacterial replicon. In each unit, DNA replication begins at a site referred to as the origin of DNA replication, and leads to the formation of a replication bubble that expands bidirectionally using two replication forks. Replication forks travel in opposite directions until they merge with forks from neighboring replication units. DNA replication originates at tens of thousands of different sites in the genome of each cell nucleus. In somatic cells, these sites are spaced at irregular intervals from 30 to 300 kb* along DNA molecules of great sequence complexity,[23,29] although in some embryonic cells, such as cleavage nuclei in *Drosophila* embryos,[4] the mean origin-to-origin distance is only 8 kb.

Identification of replication origins at unique genomic sequences has been one of the challenges of modern molecular biology. The magnitude of the problem can be appreciated by considering the difficulty in finding a single viral origin of replication integrated in a mammalian chromosome. Viral origins are all less than 1 kb in size. A 1 kb sequence of nascent DNA generated at a unique chromosomal site in randomly proliferating mammalian cells would constitute only 1 part per 20 billion parts of cellular DNA.** This is more difficult than finding one person out of the entire population of planet Earth! Never-

* Abbreviations are kb (kilobases), Thd (thymidine), dC (deoxycytidine), BrdU (bromodeoxyuridine), DHFR (dihydrofolate reductase), CHO cells (Chinese hamster ovary cells), and ADA (adendosine deaminase).

** Assuming bidirectional DNA replication and an average replication fork rate of 50 nucleotides/s, 10 s of DNA synthesis will produce about 1 kb of nascent DNA at a unique site. In a synchronized population of mammalian cells, this amount of nascent DNA will constitute 1 kb/3 × 10^6 kb total DNA of the total genome. In an unsynchronized population of cells with an average cell cycle period of 20 h, this will constitute 1/7200 of the cell cycle time. Therefore, this unique segment of nascent DNA will constitute (1/3 × 10^6)(1/7200) = 1/2.16 × 10^{-10} or about 1 part per 20 billion parts of total DNA.

1040-9238/92/$.50

theless, new experimental approaches for identification of origins of DNA replication in eukaryotic cell chromosomes have been introduced during the past several years that allow initiation sites to be mapped in single copy DNA loci in organisms as complex as mammals. However, these new approaches frequently appear complicated to those who would like to use them for the first time. Therefore, the purpose of this article is to introduce these methods in a simple manner with the hope that it will encourage their application to new systems and to novel questions.

An origin of DNA replication can be defined both functionally and genetically. Functionally, a replication origin is the physical location where DNA synthesis begins. Genetically, origins are the cis-acting sequences required for initiation of DNA replication. Thus, all experimental strategies for identification of replication origins can be classified into two groups. The first group consists of methods for physical mapping and isolation of replication origins. These methods can be subdivided into analysis of newly synthesized DNA strands and analysis of replication structures. The second group consists of functional assays for cis-acting DNA sequences conferring autonomous plasmid replication. What follows is a description of the rationale behind each of the available methods, examples of their application, as well as some of their advantages and limitations. In addition, we have attempted to provide a guide to selecting among these methods for the ones most appropriate for a particular system.

II. METHODS FOR IDENTIFICATION OF ORIGINS BASED ON ANALYSIS OF NEWLY SYNTHESIZED DNA STRANDS

A. Fiber Autoradiography

1. Rationale

Fiber autoradiography was used widely in early studies on DNA replication to elucidate the pattern of chromosomal initiation sites in eukaryotes.[23,29,41] Although this method does not map origins, the concept leads to more recent methods based on nascent DNA analysis. Replication bubbles of all possible sizes exist in unsynchronized proliferating eukaryotic cells: small bubbles represent those that have just been initiated, while large bubbles represent those whose replication forks are approaching the ends of the replicons. Most of these bubbles can be visualized if nascent cellular DNA is briefly labeled (~10 min) with radioactive precursor ^{3}H-Thd.[41] DNA is released from cells by gentle lysis, and DNA fibers are spread on a microscopic slide. Slides can be covered with radiation-sensitive emulsion and exposed until sufficiently intense silver grains are produced. The resulting autoradiogram will show dense tracks of silver grains corresponding to the nascent DNA strands of the bubbles. Bidirectional and unidirectional replication can be distinguished most easily by first using high specific radioactivity ^{3}H-TdR followed by changing to isotope-free medium for another ~10 min in order to radiolabel the same nascent DNA strands with low specific radioactivity ^{3}H-Thd. Autoradiography reveals two types of radioactive tracks: dark heavy tracks, generated by the incorporation of high specific radioactivity label, followed on the same fiber by lighter tracks corresponding to the pulse with low specific radioactivity.[41] If replication is bidirectional, two tracks will lie in register separated by a gap. If replication is unidirectional, only one track will appear. The center of the unlabeled spacer between two dark tracks indicates the position of the origin of bidirectional replication. The distance between two neighboring origins reveals the size of a particular replication unit.

2. Advantages

The results are easily interpretable, and allow detection of clusters of tandemly arranged replication origins that function during a given time interval. It is the only technique that permits analysis of the overall organization pattern of replication units on long stretches of chromosomal DNA, regardless of the frequency of replication bubbles. In principle, electron microscopy can also be used for this purpose, but it requires a high frequency of replication bubbles (e.g., *Drosophila* embryos[4]). Fiber autoradiography is useful for estimating the average replicon size. It

can distinguish between unidirectional and bidirectional DNA replication origins, and it can be applied to virtually any cell undergoing proliferation *in vivo* (i.e., cell culture conditions).

3. Limitations

The major problems are that replication origins cannot be related to specific DNA sequences, and that collection of data requires from one to several months.

B. Earliest Labeled DNA Fragment

1. Rationale

In synchronized cell populations, nascent DNA synthesized at the beginning of S-phase should come from those replication origins that are activated first.[33] Therefore, DNA fragments that are closest to an origin of DNA replication should be labeled first as cells pass from G1 into S-phase. These fragments can be identified by synchronizing mammalian cells in culture at their G1/S border and then releasing them into S-phase in the presence of a radiolabeled DNA precursor such as ^{14}C or ^{3}H-Thd. After 10 to 30 min, the cells are lysed and their DNA digested with one or more restriction endonucleases to generate specific DNA fragments (Figure 1). DNA restriction fragments are fractionated by gel electrophoresis, and those containing nascent DNA are identified by autoradiography.[33] Alternatively, pulse-labeled nascent ^{3}H-DNA can be used as a radioactive probe to identify the earliest replicating sequences by hybridizing the ^{3}H-DNA to cloned DNA fragments.[13] Both of these methods have been applied to the amplified DHFR locus in CHO C400 cells, a cell line containing 500 tandem copies of an approximately 200 kb long genomic region.[13,33]

Cultured cells are collected at their G1/S border by isoleucine deprivation followed by an aphidicolin block. The earliest replicated sequences appear as dark radioactive DNA bands against a strong background of heterogeneous labeled total DNA. Much of this background can be eliminated using the in-gel renaturation technique.[73] For this purpose, *in vivo* labeled early replicated genomic DNA sequences are mixed prior to electrophoresis with an excess of unlabeled cloned DNA sequences of interest (e.g., DHFR region). The separated DNA fragments are subjected to two rounds of denaturation-renaturation, followed by digestion with an endonuclease that digests single-stranded DNA specifically (e.g., S1). Most of the single-copy genomic DNA sequences remain single-stranded after such treatment and are subsequently eliminated by digestion with single-strand specific S1 endonuclease. In contrast, repeated DNA sequences, such as those from the amplified DHFR region, renature with the added cloned DHFR DNA sequences and thus become resistant to S1 nuclease. This technical improvement increases the resolution of origin mapping in the DHFR locus to approximately 2 kb.[54]

2. Advantages

The earliest labeled fragment strategy is one of the simplest methods for locating specific origins of replication.

3. Limitations

This method requires several hundred copies per genome of the DNA region of interest. Otherwise, it is difficult, if not impossible, to detect specific DNA fragments among the background of single copy sequences. The method works only with highly synchronized populations of cells that can be radiolabeled with DNA synthesis precursors. Thus, this method is limited to sequences that replicate at the beginning of S-phase, and because cell synchrony is never perfect, resolution is limited to several kilobases. Moreover, synchronization procedures are not only laborious, but may alter the normal pattern of initiation events. The presence of repeated sequences in the vicinity of the origin will interfere with accurately mapping an origin by hybridization unless restriction fragments are carefully selected to avoid such repeats. This is frequently difficult to do. Finally, this method does not provide any information as to the mechanism by which rep-

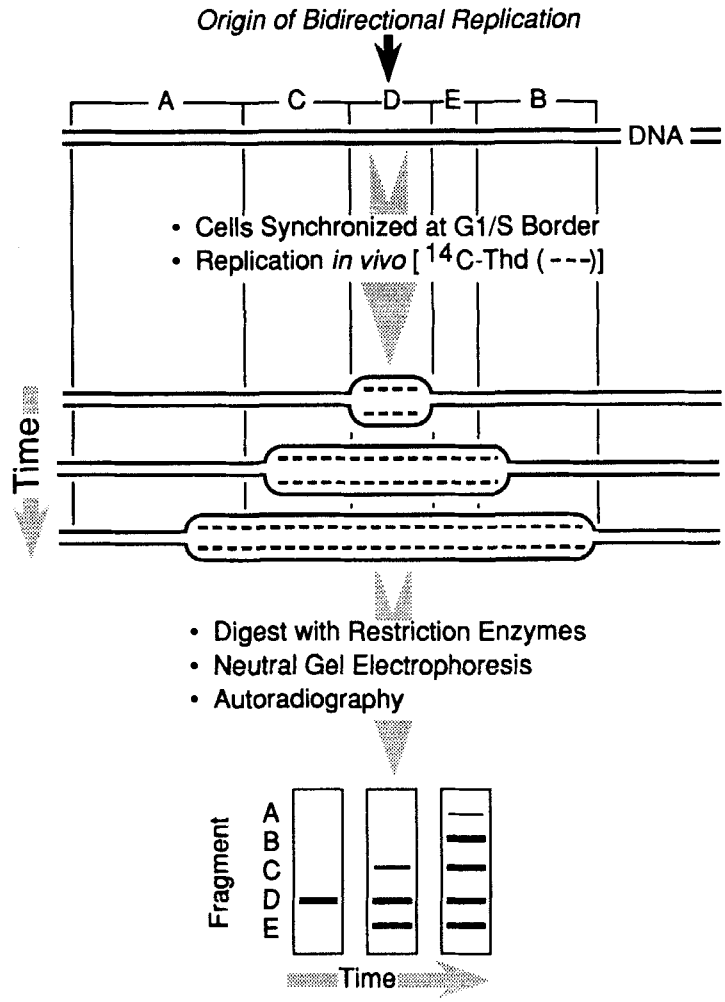

FIGURE 1. Earliest labeled DNA fragment strategy for localization of replication initiation sites in cells synchronized at the G1/S border. A, B, C, D, and E designate DNA fragments that can be generated by digestion with restriction enzymes. Dashed lines in the replication bubbles represent nascent DNA strands.

lication occurs at a particular DNA region. If nascent DNA is hybridized to cloned DNA segments, then one must take special precautions to avoid repeated sequence elements in the DNA fragments chosen (see following section on limitations for replication origin trap method).

C. Replication Origin Trap

1. Rationale

Psoralen derivatives can penetrate living cells and, on irradiation with long wave UV light, covalently cross-link their DNA. Even after relatively heavy cross-linking of DNA, cells to some extent retain their ability to incorporate radioactive precursors into nascent DNA. This allows one to trap nascent DNA at newly activated replication origins located between two neighboring cross-links (Figure 2).[77] Nascent DNA that results from replication initiated at an origin of replication positioned between two psoralen cross-links can be separated from bulk DNA by denaturation. Nascent DNA resulting from repair of damaged DNA is ligated to long DNA strands that are cross-linked with psoralen and therefore cannot be separated from bulk DNA by denatur-

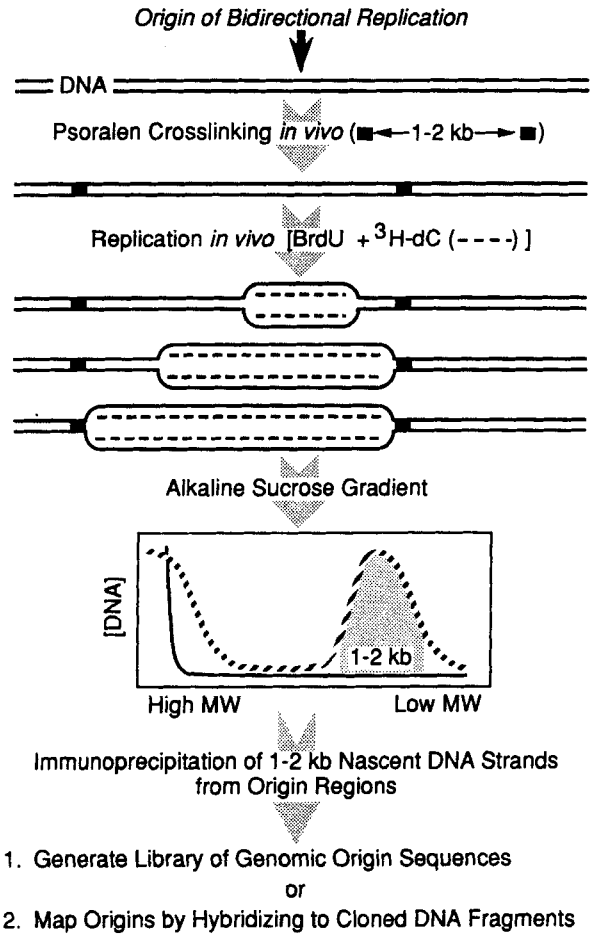

FIGURE 2. Replication origin trap strategy for isolation of origin-enriched DNA fraction from unsynchronized mammalian cells. Solid rectangles symbolize covalent psoralen cross-links in cellular DNA. Dashed lines represent nascent DNA strands synthesized between cross-links. Shaded area of the low molecular weight peak of the sucrose gradient is collected for further purification of nascent DNA chains.

ation. Nascent DNA resulting from extension of replication bubbles that are initiated prior to psoralen cross-linking will contain at least one cross-link and also cannot be separated from bulk DNA by denaturation.

Cellular DNA is cross-linked *in vivo* at approximately 2 kb intervals. The density of cross-links is determined either by electron microscopy or reassociation kinetics of fragmented cellular DNA.[77] Cells are then allowed to resume DNA synthesis in the presence of ^{3}H-dC and BrdU in order to label newly initiated DNA strands. Short nascent DNA synthesized between cross-links, which are enriched in origin sequences, is separated from the bulk of high-molecular-weight genomic DNA by centrifugation through an alkaline sucrose gradient. At this point, there are two pathways that one can follow. First, the nascent DNA fraction containing 1 to 2 kb long DNA can be converted into double stranded DNA and then cloned in order to generate a library of origin-enriched sequences. For that purpose, nascent BrdU-DNA is purified from contaminating DNA fragments in the same size group by immunoprecipitation with anti-BrdU antibodies.[94] Oligo (dA_{10-20}) tails are added to the purified nascent DNA strands. This permits synthesis of the complementary strand by annealing oligo (dT_{10-20}) primers and extending them with *E. coli* DNA polymerase I Klenow fragment. Following S1 nuclease treatment they were cloned in pBR322 as described in Sambrook et al.[78] (L. Vassilev and G. Russev, unpublished results).

Alternatively, the short nascent DNA fraction can be used as a radioactive probe to detect replication initiation sites by hybridization to cloned genomic DNA fragments. This method was applied to CHO C400 cells by Anachkova and Hamlin[1] to localize origins of replication in the amplified DHFR gene domain.

2. Advantages

This approach is applicable to any cultured cell line, and is the only available method for isolating a representative population of chromosomal origins that are activated throughout S-phase. It does not require cell synchronization, and therefore all cellular origins are equally represented in the isolated fraction of short nascent DNA, although cell synchronization can serve to enrich for a particular origin that fires at the beginning of S-phase (e.g., DHFR origin[1]). This method allows for physical isolation and cloning of origin-enriched DNA as well as identification of their specific genomic locations.

3. Limitations

If the DNA fragment chosen for hybridization with nascent DNA contains repeated DNA sequence elements (e.g., Alu sequences), an in-

creased hybridization signal will occur, regardless of whether or not this DNA fragment contains an origin of replication. This is because repeated elements are distributed widely throughout the genome and the nascent DNA fraction will contain many copies of them. Therefore, nascent DNA synthesized at one genomic location can adventitiously hybridize to other sites in the genome. To avoid this artifact, the DNA fragment chosen for hybridization should not contain repeated elements. The alternative is to prehybridize the labeled nascent DNA fraction with unlabeled repeated sequences (e.g., Alu) in order to decrease their effect in the total hybridization signal.[1] Another potential problem is that psoralen treatment of living cells may alter their physiology, causing DNA replication to initiate at sites not normally used. DNA sequences cloned by this method as putative origin sequences should be screened by one of the other methods described in this review in order to identify true origins from possible contaminating DNA sequences.

D. Nascent Strand Extrusion

1. Rationale

Replication bubbles in nicked or linear SV40 DNA are unstable at elevated temperatures,[102] and therefore, under appropriate conditions, nascent DNA strands can be extruded selectively from replication bubbles. Extrusion is a type of branch migration in which nascent DNA strands within replication bubbles are transiently released from their templates and anneal with one another to form double-stranded DNA fragments. If nascent DNA is extruded from small replication bubbles, a substantial amount of its sequence should represent the origin of replication. One way to enrich for such early replication intermediates is to isolate DNA from synchronized cells that have just entered S-phase. Purification of the extruded DNA from the vast excess of total contaminating DNA would make possible its cloning and generation of a library of replication initiation sequences.

An experimental protocol (Figure 3) has been designed that allows for isolation and cloning of short extruded origin-enriched DNA fractions from mammalian cells.[46] Cells are first collected at their G1/S border by a combination of serum deprivation and arresting DNA synthesis with aphidicolin, and then released into S-phase for 1 min in the presence of ^{3}H-Thd to label newly synthesized DNA. Nuclei are then isolated and DNA synthesis continued in the presence of Hg-dCTP to add an affinity label to the nascent DNA strands. Since synthesis *in vitro* is inefficient relative to synthesis *in vivo*, Hg-dCTP is generally incorporated for an additional 10 min. Thus, DNA chains that were initiated at origins activated at the beginning of S-phase should now contain both a radiolabel for easy identification as well as an affinity label for easy purification by affinity chromatography on sulfhydryl-sepharose column. Extrusion is carried out for 4 to 6 h at 50°C in 0.2 *M* Tris-HCl (pH 7.5) and 20 m*M* EDTA.

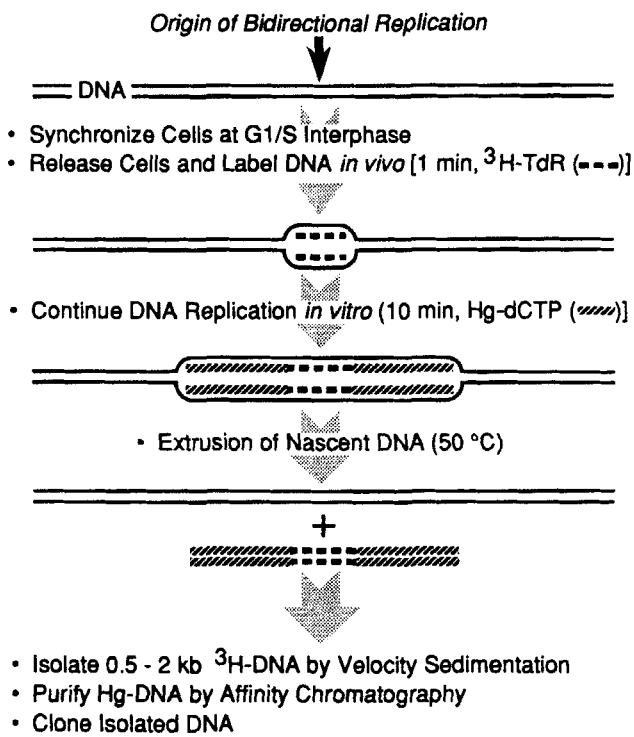

FIGURE 3. Nascent strand extrusion strategy for isolation and cloning of origin-enriched mammalian DNA fraction.

Nascent DNA chains extruded from replication bubbles are complementary and therefore should produce double-stranded linear DNA molecules. Small-molecular-weight DNA (average size 0.5 to 2 kb), which should be enriched for origins, is isolated by sucrose gradient centrifugation. This fraction is then treated with a single strand specific endonuclease (e.g., S1) to produce double stranded DNA with blunt ends. These molecules are cloned,[46] and several such cloned DNA segments have been reported to support autonomous replication in human cell lines.[25]

A modification of this technique has been used to map the replication initiation region of the chicken α-globin gene.[71] In this study nascent DNA strands are labeled with BrdU so that after extrusion they can be purified from unreplicated DNA by centrifugation to equilibrium in a CsCl gradient. Short (3 to 5 kb) nascent BrdU-DNA, derived from relatively small replication bubbles and thus enriched in origins of replication, are end-labeled with ^{32}P *in vitro* and used as a hybridization probe to screen cloned DNA fragments from the α-globin gene locus. This putative initiation locus is localized within a 5-kb DNA fragment upstream of the transcription start site.

2. Advantages

This method allows isolation of origin sequences from virtually any cultured mammalian cell line.

3. Limitations

This method is limited to initiation sequences active at the onset of S-phase. As with the replication origin trap method, it is impossible to distinguish between cloned fragments containing genuine initiation sites and fragments containing contaminating genomic DNA. Therefore, it is necessary to demonstrate that the cloned DNA sequences exhibit origin function. One potential problem with this method is incorporation of Hg-dCTP by repair during *in vitro* DNA synthesis.

E. Nascent DNA Strand Length

1. PCR Approach

a. Rationale

In an asynchronous population of proliferating mammalian cells, the size of replication bubbles generated by each origin of replication will vary from small bubbles that had just been initiated to large bubbles that are about to complete replication. If one can identify the shortest nascent DNA strands that contain a unique DNA sequence, then one can map the origin of replication for those nascent strands.[92] If replication is bidirectional, the origin of replication is at the center of the strands. If replication is unidirectional, then the origin is at one end.

To map an origin, a minimum of three unique DNA segments are selected that are distributed across the putative origin region (e.g., segments A, B, and C in Figure 4). These segments should not contain repeated DNA elements. Nascent DNA chains are then radiolabeled for 10 min *in vivo* with ^{3}H-dC to allow their convenient detection and with BrdU to allow their isolation from unreplicated DNA. This is sufficient time to produce BrdU-labeled regions at least 20 kb in length. Nascent DNA is then fractionated according to length under denaturing conditions. This can be done either by sedimentation through alkaline sucrose gradients[92] or by electrophoresis in alkaline agarose gels (V. Virta-Pearlman and C. Chinault, unpublished data). Newly replicated BrdU-DNA in each fraction can be purified from contaminating unreplicated DNA by immunoprecipitation with anti-BrdU antibodies.[94] Alternatively, the whole population of nascent BrdU-DNA strands can be purified prior to fractionation according to chain length by sedimentation to equilibrium in an alkaline CsCl gradient (V. Virta-Pearlman and C. Chinault, unpublished data).

To detect single-copy sequences, oligonucleotides are synthesized complementary to each of the unique DNA segments selected. These will serve as primers for specific amplification of the designated segments by the polymerase chain reaction (PCR) technique. PCR amplification in the

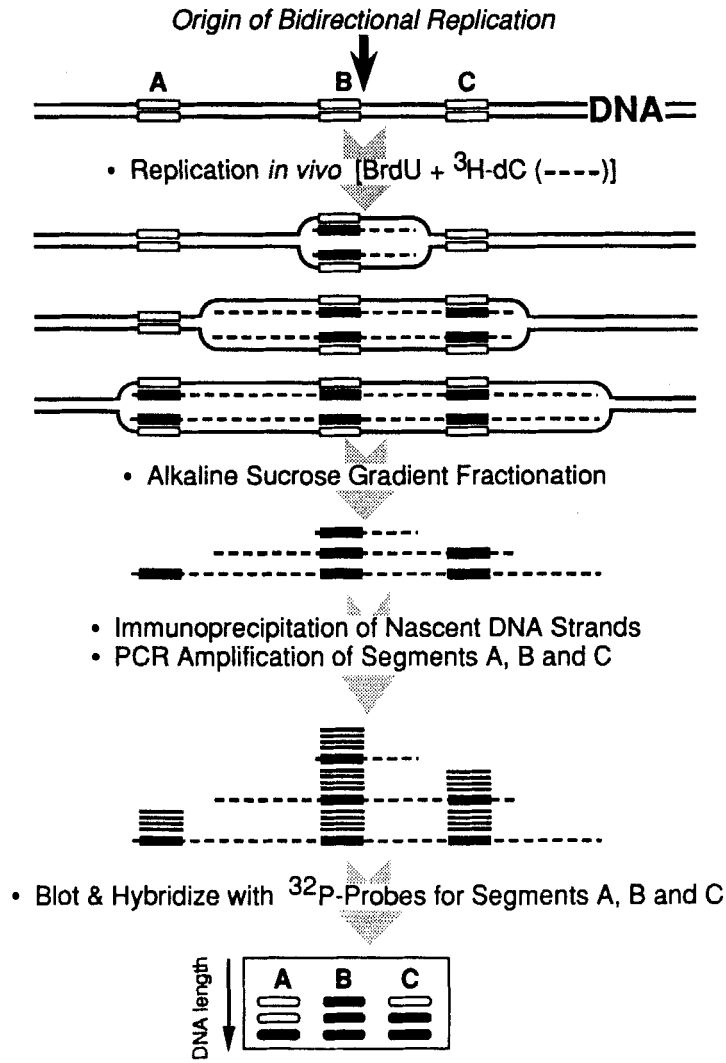

FIGURE 4. Nascent DNA strand length PCR approach for mapping origins of DNA replication. Adapted from Vassilev et al., 1990.

presence of all the primers will raise the concentration of the selected DNA segments to easily detectable levels. The size of the PCR products should be checked by gel electrophoresis to confirm their expected lengths. Detection is usually done by transferring the PCR products to a membrane and then hybridizing with synthetic oligonucleotide ^{32}P-probes complementary to the unique DNA segments. However, if the sequence under examination is already amplified several hundred fold (e.g., cells that contain an amplified gene), then the PCR products may be visualized by subjecting them to gel electrophoresis and staining with ethidium bromide (V. Virta-Pearlman and C. Chinault, unpublished data).

Depending on their initial length, nascent DNA strands will contain one or all of the unique sequences selected. If the origin lies outside all of the segments, then the number of copies of each segment present in the PCR products will reveal forks progressing from one end to the other. In that case, a new set of unique sequence markers must be selected further upstream. If the origin lies between two segments (as shown in Figure 4), then the shortest nascent chains will contain only that segment closest to the origin, while the longer nascent chains will contain the segment closest to the origin plus one or more of the other segments selected. If replication is bidirectional, the nascent DNA chains will contain DNA seg-

ments from both sides of the origin with the ratio of B/A and B/C decreasing as shorter chains grow into longer chains. The size of the shortest DNA fraction that contains a given segment is determined as described by Vassilev et al.,[95] and from this distance the location of the origin is calculated.

This mapping approach has localized an origin of bidirectional replication in single copy sequences of the c-*myc* gene locus in human cells,[93] the DHFR gene locus in CHO cells,[95] and the ADA gene locus in mouse cells (V. Virta-Pearlman and C. Chinault, unpublished data).

b. Advantages

This is the most sensitive origin mapping technique developed so far. It is generally applicable to any single copy locus in randomly proliferating mammalian cells. It can detect both bidirectional and unidirectional origins of replication. The technique avoids the use of metabolic inhibitors and cell synchronization allowing origin function to be studied under physiological conditions. If an origin has repeated sequences nearby, one can still apply this technique by simply selecting PCR primer sites that lie outside of these repeats. Origins can be mapped within 2 to 3 kb. Because this method only isolates nascent DNA strands <10 kb, it does not detect replication forks from neighboring replicons that might be traveling through the region of interest. Therefore, this method is capable of mapping origins that fire less than once per cell cycle.

c. Limitations

This method is too laborious for exploring long genomic DNA regions for origins. It is most suitable for mapping an origin in DNA regions <10 kb where the existence of an origin has been suggested previously by other data. Sequence information is required for the synthesis of appropriate PCR primers. This technique is limited to DNA segments not farther than 5 to 6 kb from an origin because of the light sensitivity of nascent BrdU-DNA chains and the inaccuracy in fractionation of long DNA chains into distinct size classes. If a PCR segment lies too close to repeated genomic elements (e.g., Alu sequences), then the rate of reannealing BrdU-nascent DNA chains with unreplicated DNA of the same sequence will be increased during the time required for immunoprecipitation. This artifact could produce a higher PCR signal from that segment.[93] This problem can be avoided by proper selection of amplification segments or use of the equilibrium density centrifugation step for purification of BrdU-DNA (V. Virta-Pearlman and C. Chinault, unpublished results).

2. Kinetic Approach

A second variation of the nascent strand length strategy is to synchronize cells in order to measure nascent DNA length growth as a function of time. Then the size of the shortest nascent DNA fragment detected by a specific probe indicates the approximate distance between that probe and the origin of replication. This approach has been applied to *Physarum polycephalum*[3] (Figure 5). Physarum plasmodia contain many nuclei that are naturally synchronized, and its genome complexity is near 100-fold less than mammals that allows detection of replication intermediates from single copy sequences by hybridization without first amplifying the sequences using PCR. Unlabeled nascent DNA synthesized in the first minutes of S-phase can be separated from the bulk of cellular DNA by denaturing gel electrophoresis. DNA template strands as well as unreplicated regions of DNA will remain at the top of the gel because of their length, whereas short nascent DNA chains from newly initiated origins will migrate into the gel. Nascent DNA is distinguished from template DNA and from unreplicated DNA because nascent DNA is short enough to enter the gel, and its length increases with time after the cells have entered S-phase, whereas template and unreplicated DNA are too long to enter the gel at any time during S-phase. Following gel electrophoresis, DNA is transferred to a membrane and then hybridized with specific ^{32}P-DNA segments representing unique sequences in the region of the putative origin. The probe that anneals with the shortest DNA chains is the probe that lies closest to the origin

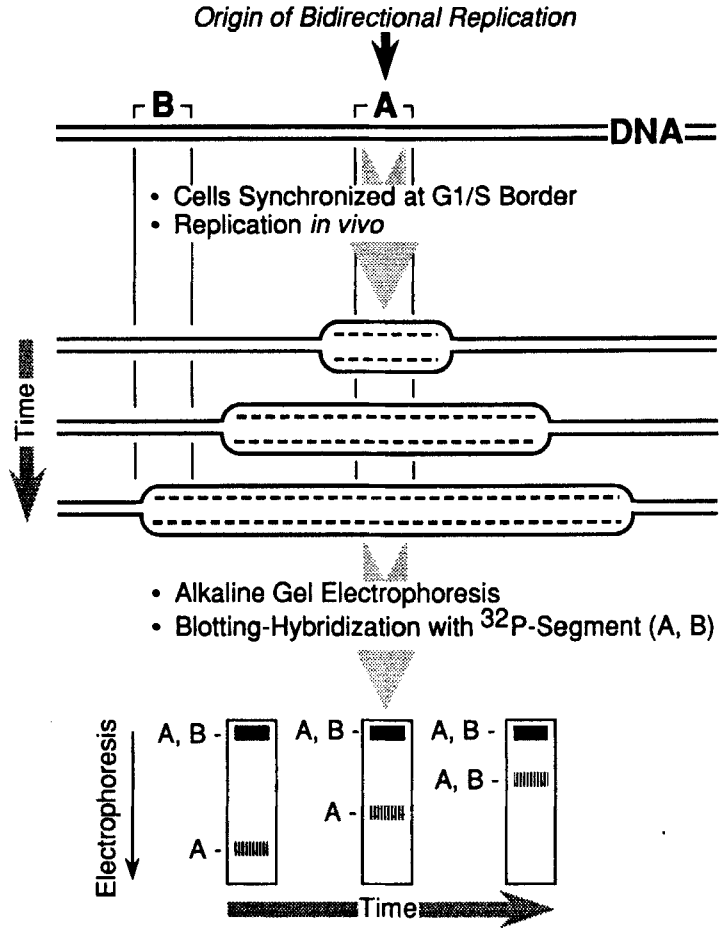

FIGURE 5. Nascent DNA strand length kinetic approach for mapping origins of DNA replication (adapted from Benard and Pierron, 1990). Locating an origin of replication by measuring growth of nascent DNA chains in synchronous plasmodia of *Physarum*. Nascent DNA chains are represented by dashed lines. Solid rectangles symbolize unreplicated DNA in the high molecular weight region of the agarose gel.

of replication. Using this method, an origin of replication was detected within about 5 kb of a DNA fragment containing the LAV1-2 gene.[3]

a. Advantages

The technique is relatively simple.

b. Limitations

This approach can be applied only to highly synchronous cells with genomes of low complexity such as the plasmodium of *Physarum*. Any slight asynchrony will significantly decrease the resolution and will make the technique impractical.

3. Run-Off Replication Approach

a. Rationale

Initiation of DNA replication does not appear to occur in isolated nuclei, although replication bubbles that are initiated *in vivo* can continue replication following cell lysis. Therefore, when nuclei are isolated from an asynchronous population of proliferating cells, they contain sets of

replication bubbles of various sizes that were initiated at particular origins. At any given origin, the smallest bubbles have replication forks closest to the origin, while those bubbles that have reached the ends of the replicon will be the largest. Thus, the direction of replication through a unique DNA sequence can be determined by extending nascent DNA chains in this region in the presence of BUdR. The direction of replication is deduced based on the relative amount of DNA synthesis at several different loci.[45] In the absence of initiation, those fragments furthest from the origin will incorporate the greatest amounts of density label. The amount of incorporated label complementary for a unique DNA sequence will increase proportionally as the distance between this sequence and the origin of replication increases because the number of nascent DNA chains that can be extended through this sequence will be greater.

Isolated nuclei from exponentially proliferating mammalian cells are digested with an appropriate restriction enzyme and incubated *in vitro* in the presence of BrdUTP under conditions permitting the completion of the elongation of those replication bubbles, initiated *in vivo*, that had passed the restriction site. All the nascent DNA strands from the replication bubbles that have not reached the restriction sites will run off the cleaved template. Thus, restriction digestion prior to *in vitro* labeling emphasizes the difference in the labeling of two segments, one located in the vicinity of origin and the other further away. Cellular DNA is then digested to completion by appropriate restriction endonucleases fragmenting the unique sequence of interest. BrdU-labeled (heavy) fragments are separated from the unreplicated (light) DNA by equilibrium CsCl gradient centrifugation. Blotting and hybridization with each ^{32}P-labeled restriction fragment is used to determine its relative concentration in the fraction of BrdU-DNA, and therefore the polarity of replication through the locus. Restriction fragments showing relative enrichment in the fraction of *in vitro* added DNA chains are distal to an active origin of replication. The polarity of replication and approximate location of the initiation site can be deduced by examining pairs of DNA segments divided by a restriction cut and introduced in the template DNA prior to *in vitro* extension, spanning several kilobases in both directions from it.[62]

The method has been used to measure replication polarity in the avian α-globin locus[45] and the results are consistent with the α-globin origin mapped by the nascent strand extrusion described previously.[71] This method has also been used to map an origin upstream of the human c-*myc* locus to within 3.5 kb,[62] consistent with results from the PCR approach described previously.[93]

b. Advantages

The method is applicable to any cell line. It is sufficiently sensitive to detect replication intermediates of single copy sequences in asynchronous mammalian cells. Replication is initiated *in vivo* under normal physiological conditions.

c. Limitations

Although useful for determination of the direction of replication through single-copy DNA loci, this technique has not yet proven practical for mapping initiation sites. Hybridization signals from single copy mammalian loci are weak and resolution is low.

F. Replication-Fork Polarity

1. Okazaki Fragment Distribution Approach

a. Rationale

Assuming that DNA replication in mammalian chromosomes is initiated bidirectionally at a specific site using the replication fork model, an origin of bidirectional DNA replication (OBR) can be identified by the transition from discontinuous to continuous DNA synthesis that must occur on each template strand at the site where replication forks originate.[31] This results from synthesis of Okazaki fragments predominantly, if not exclusively, on the retrograde arms of forks

(Figure 6). In discontinuous DNA synthesis, Okazaki fragments are repeatedly initiated, elongated, and joined to the 5′-ends of long nascent DNA strands. Usually the ligation step occurs within 1 min of completing synthesis,[89] resulting in long nascent DNA strands labeled at both ends. Therefore, when DNA is sonicated prior to hybridization, the labeled 3′-end of long DNA chains will hybridize to one template strand, while the labeled 5′-end will hybridize to the other template strand. In contrast, the replication fork model predicts that Okazaki fragments will anneal predominantly, if not exclusively, to the retrograde arm under all conditions of labeling. In addition, Okazaki fragments are easily separated from the large excess of unlabeled DNA that interferes with hybridization of ^{32}P-DNA to immobilized DNA by annealing with the ^{32}P-DNA in solution. Thus, hybridization of Okazaki fragments to cloned strand-specific DNA templates that span the initiation site will reveal the expected transitions between discontinuous and continuous DNA synthesis, and thus can be used to identify an OBR.

Cells must be synchronized at their G1/S border in order to accumulate a large number of replication forks within the initiation zone. In an asynchronous population of mammalian cells, the quantity of Okazaki fragments that could be isolated from a reasonable number of cells is insufficient to detect a hybridization signal. Okazaki fragments are labeled for 1.5 min by treating the cells with NP-40 to make them permeable to dNTPs, and then they are released into S-phase in the presence of [α-^{32}P]dATP and BrdUTP under conditions that permit faithful continuation of DNA replication events initiated *in vivo*.[12] The same early labeled fragment observed by labeling nascent DNA in intact cells[33] is also labeled first in NP-40 treated cells[12,13] and in isolated nuclei.[34]

Okazaki fragments are distinguished from DNA repair products in four ways:[19–21] (1) Okazaki fragments are short nascent DNA fragments with a mean length of 100 to 150 nucleotides; (2) Okazaki fragments are rapidly joined to long, growing nascent DNA strands; (3) Okazaki fragments have RNA primers (oligoribonucleotides 8 to 12 residues long) covalently attached to

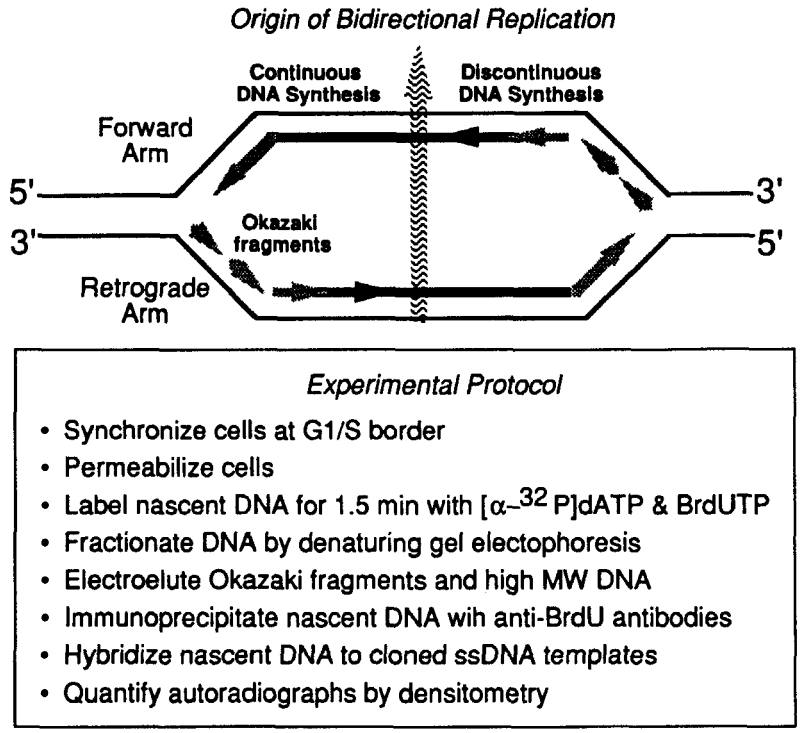

FIGURE 6. Okazaki fragment distribution strategy for mapping origins of bidirectional DNA replication. (Adapted from Burhans et al., 1990.)

their 5′-ends; (4) Okazaki fragments anneal predominantly, if not exclusively, to DNA templates representing the retrograde arms of replication forks. All four criteria have been demonstrated for the Okazaki fragments used to map SV40,[31] polyomavirus,[37] and the hamster cell DHFR OBRs.[10–12]

At least three factors are critical in obtaining a significant amount of radiolabeled Okazaki fragments. The labeling period must be equal to or less than the time required to synthesize an Okazaki fragment and ligate it to the 5′-end of a growing nascent DNA strand. The rate of DNA synthesis and ligation is strongly temperature dependent. Ligation of Okazaki fragments is strongly inhibited in nuclei that have been washed free of cytoplasm, although excision of their RNA primers continues.[19–21] Purified Okazaki fragments can be radiolabeled specifically at their RNA-*p*-DNA junction,[31] but it is unlikely that this approach will yield sufficient ^{32}P-labeled Okazaki fragments to map an OBR in mammalian chromosomes.

To obtain highly purified populations of Okazaki fragments, DNA is fractionated according to its size by gel electrophoresis, recovered by electroelution, and ^{32}P-DNA is separated from unlabeled parental DNA by immunoprecipitating single-stranded Br-DNA with anti-BrdU antibodies.[94] This purification scheme eliminates unlabeled parental DNA strands that could interfere with subsequent hybridization. Nascent DNA is then hybridized to individual complementary template strands that are cloned into single-stranded bacteriophage M13 DNA and immobilized on membranes (dot-blots). The orientation of template strands is determined both from the directionality of restriction fragments used in cloning and through confirmation by sequence analysis of the cloned DNA. Hybridization signals are quantified either by scanning densitometry of autoradiograms or by scanning the membrane directly with a radiation detection instrument such as the BetaScope 603 blot analyzer (Betagen). Remember that densitometry of autoradiograms requires that the intensity of the image on the autoradiogram be proportional to the amount of radiation. Therefore, scanning must be done on several different film exposures, and known amounts of ^{32}P should be included as to generate a standard curve that ensures accuracy. With high-energy-emitting isotopes such as ^{32}P, it is not necessary to preexpose film to achieve a linear response, although preexposing film in addition to using an intensifying screen may increase sensitivity.[53]

Results from this approach identified an OBR within a 450-bp region 17 kb downstream from the DHFR gene in both single copy CHO cells and CHO C400 cells containing amplified DHFR genes (Figure 6).[12] At least 80% of the replication forks in a 27-kb region emanated from this OBR. We refer to this site as DHFR OBR-1. Similar results have been obtained at the amplified CAD gene complex in mouse cells[14] (R. Kelly and G. Wahl, unpublished data). This same strategy for locating an OBR, when combined with cutting the Okazaki fragment ^{32}P-DNA:template DNA hybrids at a unique restriction site, has been used to map the OBR with single nucleotide resolution in SV40,[28,31] polyomavirus,[37] *E. coli*,[48,74,82] and bacteriophage λ.[101] In each case, the viral and bacterial OBRs were found close to or coincident with their genetically required origin sequences. Thus, initiation of DNA replication in mammalian chromosomes uses the same replication fork mechanism described previously in a variety of prokaryotic and eukaryotic genomes, suggesting that mammalian chromosomes also utilize specific cis-acting sequences as origins of replication.

b. Advantages

This technique is arguably the most well understood in terms of its theoretical basis, and it offers the greatest precision for mapping an OBR. That, however, depends on availability of unique probes and appropriate restriction sites in the vicinity of the origin. The resolution achieved so far in mammalian cells is 450 bp.[12] This technique can also be used as an assay for origin function. Unique hybridization probes from any segment within a given replicon should work equally well.

c. Limitations

This technique only applies to genomes using the replication fork mechanism, and is applicable

only when Okazaki fragments can be shown to anneal preferentially to one template strand. If replication forks from origins that lie in adjacent regions pass through the region of interest, then the predicted strand bias for Okazaki fragments will be erased. This problem will be most serious in populations of unsynchronized cells. This, together with limitations on detecting Okazaki fragments from a unique single-copy sequence, limits application of the method to mammalian replication origins that are activated at the onset of S-phase. Therefore, cells must be synchronized with the help of metabolic inhibitors such as aphidicolin and DNA radiolabeled in cell lysates. These treatments may alter the process of DNA replication.

2. Imbalanced DNA Synthesis Approach

a. Rationale

Handeli et al.[30] reported a method for mapping an OBR by analyzing DNA synthesis on forward arms of replication forks after treating exponentially proliferating cells with emetine, a potent inhibitor of protein synthesis. Early studies suggested that, in the absence of histone synthesis, histone octamers in front of replication forks segregate exclusively to forward arms of replication forks, leaving nascent DNA on retrograde arms unprotected by histones and therefore sensitive to nonspecific endonucleases.[72,75,80] If this were true, then an OBR could be recognized by the transition from nuclease protected to nuclease sensitive nascent DNA on each template strand, analogous to measuring the transition from continuous to discontinuous DNA synthesis. When Handeli et al.[30] applied this approach to the DHFR locus, they concluded that the bulk of replication forks downstream of the DHFR gene in CHO cells emanated from an OBR somewhere within the 14-kb region residing between the two probes flanking the DHFR OBR identified by Burhans et al.[12] and the DHFR initiation zone identified by Vassilev et al.[95] A more recent study has confirmed this result and therefore the validity of this method for mapping origins. However, the mechanism does not involve nucleosome segregation, but rather imbalanced DNA synthesis.

In the presence of emetine, nascent DNA on forward arms of replication forks in hamster cell lines containing either single or amplified copies of the DHFR gene region was enriched 5- to 7-fold over nascent DNA on retrograde arms.[10] This forward-arm bias was observed on both sides of the specific OBR located 17 kb downstream of the hamster DHFR gene,[12] consistent with at least 85% of replication forks within this region emanating from this site. These results were entirely consistent with those of Handeli et al.[30] However, the replication fork asymmetry induced by emetine does not result from conservative nucleosome segregation, as previously believed,[30] but from preferentially inhibiting Okazaki fragment synthesis on retrograde arms of forks to produce imbalanced DNA synthesis. Three lines of evidence support this conclusion. First, the bias existed in long nascent DNA strands prior to nuclease digestion of nonnucleosomal DNA. Second, the fraction of RNA-primed Okazaki fragments was rapidly diminished. Third, electron microscopic analysis of SV40 DNA replicating in the presence of emetine revealed forks with single-stranded DNA on one arm, and nucleosomes randomly distributed to both arms. Thus, as with cycloheximide,[16,84] nucleosome segregation in the presence of emetine is distributive.

The imbalanced DNA synthesis method begins by incubating randomly proliferating mammalian cells with emetine to inhibit protein synthesis and BrdU to label DNA synthesized in the absence of protein synthesis. Under these conditions, synthesis of Okazaki fragments is inhibited 90% in the first 1 h of incubation, and total DNA synthesis is inhibited 90% by 10 h of incubation. Cellular DNA is then isolated and sonicated in order to separate labeled (heavy) from unlabeled (light) regions on the same DNA strand. Heavy DNA is separated from light DNA by equilibrium centrifugation in alkaline Cs_2SO_4 gradients. Heavy DNA and light DNA are then immobilized on a membrane and hybridized with strand-specific ^{32}P-labeled RNA probes representing specific segments of DNA throughout the

genomic region of interest. As with the Okazaki fragment method, the OBR is recognized by the transition from forward to retrograde template on each DNA strand (Figure 7). On one side of the OBR, heavy DNA representing DNA synthesis on forward-arm templates will anneal to the Watson strand while on the other side of the OBR, it will anneal to the Crick strand.

b. Advantages

This method is technically easier to execute than the Okazaki fragment distribution method. It does not require cell synchronization, and therefore can be used with virtually any origin regardless of when it is activated during S-phase. Nascent DNA is labeled *in vivo*. The method works with single copy sequences. This method can be used to assay for origin activity with probes located far away from the origin as long as the probes lie within the same replicon (i.e., as long as forks from other origins do not pass through the probes). The technique is useful for mapping the boundaries of replication units on long stretches of chromosomal DNA.

c. Limitations

The technique requires inhibition of protein synthesis for extended periods of time, which might alter the pattern or mechanism of DNA replication. Furthermore, if emetine blocks initiation of replication, then the amount of nascent DNA available will decrease as one probes closer

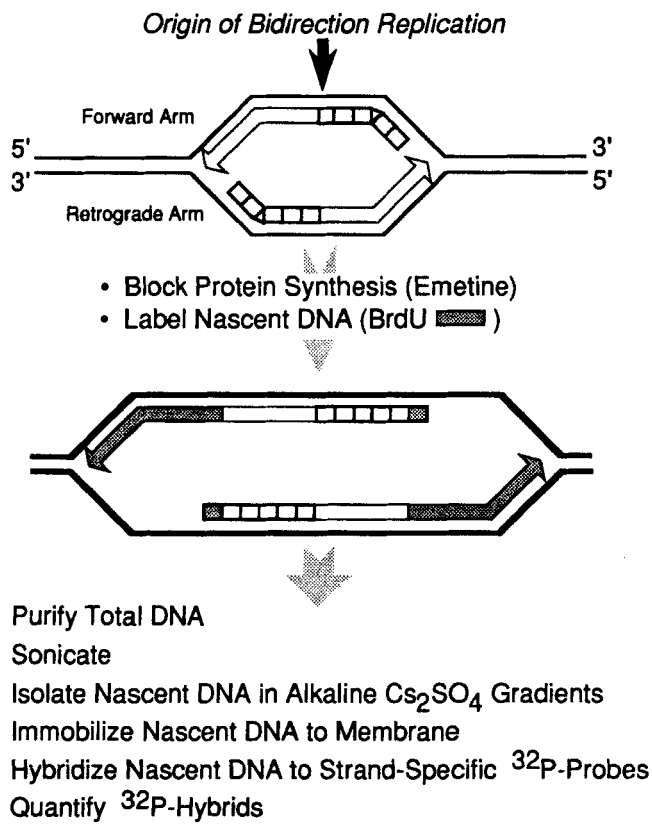

FIGURE 7. Imbalanced DNA synthesis strategy for mapping origins of bidirectional DNA replication. Small rectangles in the replication bubble represent retrograde (lagging) DNA strands formed by repeated ligation of Okazaki fragments. (Adapted from Burhans et al., 1991.)

to the origin. This will limit the sensitivity of detection. The best resolution reported so far is ~6 kb at the hamster APRT locus.[30]

III. METHODS FOR IDENTIFICATION OF ORIGINS BASED ON ANALYSIS OF DNA STRUCTURES

A. Electron Microscopy

1. Rationale

Replication bubbles are relatively simple to identify by electron microscopy. By examining two or more restriction fragments, one can map the center of the bubble relative to a unique DNA site. In this way, it can be determined if the bubbles are expanding in one or both directions. If the center of the bubble is associated with a specific sequence, then replication is bidirectional. If the center of the bubble changes position, then replication is occurring only in the direction in which the center of the bubble is moving. In the case of plasmid, bacteriophage, and animal virus DNA, this mapping strategy has proven capable of locating origins of replication within ± 100 bp.[49] Electron microscopy has been used to identify the sites where DNA replication originates in the tandemly repeated ribosomal DNA gene loci (rDNA) in *Tetrahymena*,[15,88] *Physarum*,[98] *Xenopus*,[7] and sea urchin.[5] These origins are all localized within the nontranscribed spacer regions.

2. Advantages

Electron microscopy allows direct visualization of initiation sites (replication bubbles) and their precise localization in a DNA molecule.

3. Limitations

Replication bubbles in most eukaryotic cells are difficult to find because their frequency is low (~11% of total mammalian cell DNA*), most of the larger bubbles will be lost when DNA is digested with restriction enzymes, and many of the smaller bubbles will be lost during purification of cellular DNA by extrusion of nascent DNA strands.[102] Electron microscopic mapping of origins may be feasible in *Drosophila* cleavage stage embryos[4] or yeast cells[68] where the fraction of replication bubbles is greater. In general, electron microscopy appears to be practical only in the case of repeated DNA sequences that can be isolated from total DNA. For example, rDNA genes are multiple tandem repeats (more than 200) with a higher than average GC content that allows them to be separated from total DNA by equilibrium density gradient centrifugation. Variation of the repeated length could create problems, and replication structures frequently are difficult to interpret due to possible artifacts that might occur during DNA spreading. Microbubbles (1 to 3 μm replication bubbles) may represent one example of such an artifact.[63]

B. Two-Dimensional Gel Electrophoresis

1. Rationale

The object of 2-D gel electrophoresis methods is to cut the total population of cellular DNA into unique restriction fragments and then to fractionate these fragments on the basis of their size and shape. Unique DNA sequences are identified by hybridization with specific ^{32}P-DNA probes. Replication intermediates are identified on the basis of theoretical considerations together with analysis of simple model systems such as replicating plasmid DNA.

Appropriate electrophoretic conditions can increase the contribution of the shape of a molecule to its mobility in agarose gels.[2,8] In lower concentrations of gel and at lower voltage, the mobility of DNA depends primarily on its mass. These conditions constitute the first dimension of electrophoresis. In higher concentrations of gel and at higher voltage, the mobility of DNA depends primarily on its shape. These conditions constitute the second dimension. Control experiments have shown that each type replication in-

* If there are ~4000 bubbles with a mean size of 50 μ present at any one time during S-phase,[23,29] then (50 μ/bubble)(cell/1.8 m DNA)(m/10^6 μ)(4000 bubbles/cell)(100) = ~11%.

termediate is characterized by a specific 2-D gel electrophoretic pattern. In neutral-neutral gels, DNA fragments containing (1) a single replication fork, (2) a replication bubble, (3) two replication forks, or (4) a replication bubble that eventually runs past the restriction site give characteristic patterns (Figure 8). These patterns are commonly referred to as (1) fork arcs, (2) bubble arcs, and (3) double Y. Fork arcs represent replication forks. Bubble arcs should contain an origin of replication. Double Y fragments are diagnostic for a termination site. Asymmetric bubble arks (panel 4) could indicate the position of the replicon initiation site relative to different restriction sites.

A different version of the 2-D gel method[40,66] separates replication intermediates by mass in the first dimension (neutral pH) and by nascent DNA strand length in the second dimension (alkaline pH). In this method (neutral-alkaline gels), nascent DNA strands form characteristic ark patterns that are diagnostic of the position of each restriction fragment relative to an active origin of replication (Figure 9). Fragments close to the origin give a complete ark composed of nascent strands of all sizes, while fragments distal to it would give a small ark in the high molecular weight region of the gel. Using several probes one can determine the direction of fork travel through the DNA locus.

The 2-D gel method has been reviewed recently in detail.[9,24] A modification has been developed that allows enrichment for replication intermediates from mammalian cells.[22] Replication intermediates can be enriched significantly in the cellular DNA by isolating the nuclear matrix fraction. This modification permits mapping of initiation sites in higher eukaryotes at the level of single copy sequences.

The 2-D gel methods have been used in a number of studies for mapping origins of replication in plasmids,[8,40,60] Epstein-Barr virus,[26] Bovine papilloma virus,[99] yeast chromosomes,[9,42,56] *Drosophila* chorion gene loci,[18,32,83] and hamster DHFR gene loci.[22,96,97]

2. Advantages

2-D gel methods are techniques of choice for mapping origins of replication in genomes with low complexity and genomes that do not take up DNA synthesis precursors readily. They do not involve labeling of nascent DNA. Cells do not have to be synchronized and therefore replication intermediates are isolated from cells replicating

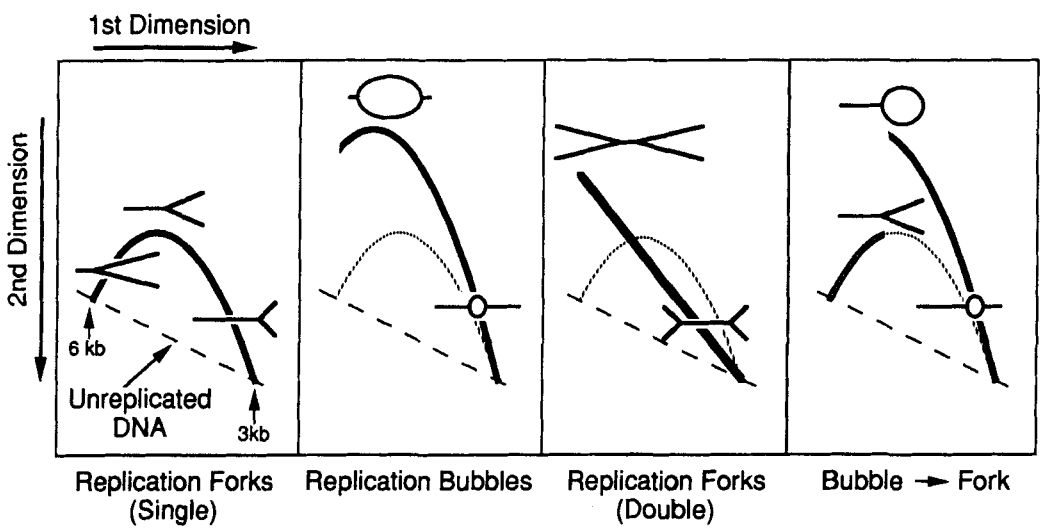

FIGURE 8. Two-dimensional gel electrophoresis strategy for mapping origins of DNA replication using neutral pH in both dimensions (adapted from Brewer and Fangman, 1987). Four typical groups of replication intermediates and their corresponding gel patterns are represented by solid lines. Dashed lines show the position of unreplicated total DNA fragmented with restriction enzymes.

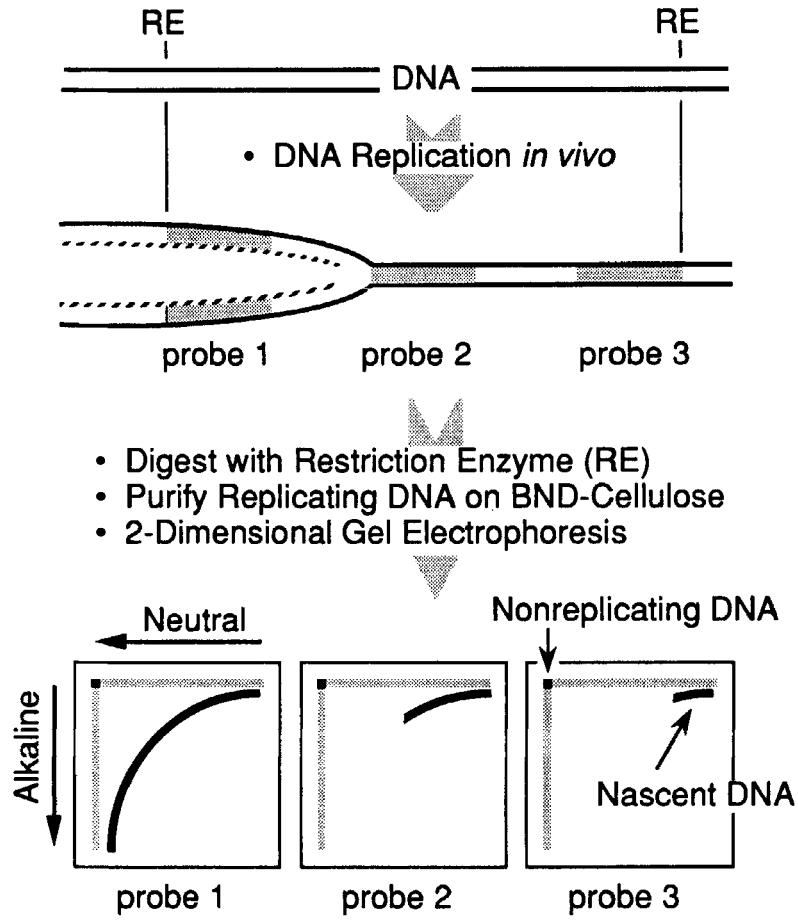

FIGURE 9. Two-dimensional gel electrophoresis strategy for mapping origins of DNA replication using neutral pH in one dimension and alkaline pH in the other. (Adapted from Nowotka and Huberman, 1988.)

under normal cell culture conditions. In BPV,[99] the origin of replication was localized to ± 100 bp. In yeast cellular DNA, origins have been mapped to several hundred base pairs, depending on the size of the DNA restriction fragments that can be produced. The appearance of replication bubbles in the 2-D gel can also be used simply as an assay for origin function in plasmids or cellular chromosomes.

3. Limitations

The 2-D gel method searches for structures that migrate as replication intermediates regardless of whether or not they contain nascent DNA. Therefore, it is possible to imagine artifacts in which unusual DNA structures may be misidentified as replication bubbles. For example, replication forks in which the DNA remains bound to cellular material may migrate anomalously slow during gel electrophoresis, and thus appear as replication bubbles. One problem with application of this method to eukaryotes with a genome complexity higher than that of *Drosophila* is that replication bubbles are undetectable unless replicating DNA is enriched first by isolating nuclear matrix material.[22] DNA associated with nuclear matrix material may enrich for replication forks in which two arms are adventitiously associated by some cellular material. These structures could masquerade as replication bubbles in the neutral-neutral 2-D gel method. Perhaps they would be detectable by comparing results from neutral-neutral gels with those from neutral-alkaline gels. In any event, enrichment for replication bubbles

by association with nuclear matrix complicates interpretation of the data because of a limited understanding of DNA-matrix interactions and their relevance to DNA replication.

It appears difficult to quantify the number of initiation events in any given DNA region of the gel. First, it is important to adjust the conditions of electrophoresis for DNA fragments >5 kb because of artifactually distorted Y-arcs and unrecognizable bubble arcs.[42] While the ratio of bubbles to forks observed in yeast experiments is close to unity, the ratio observed in mammalian cell experiments varied from ~1 to ~30%. Breaks in the replication bubbles introduced during DNA preparation could result in an underestimation of active origins of replication in the neutral-neutral 2-D technique.[55,60] It is also difficult to distinguish a symmetrically located origin of bidirectional replication from an asymmetrically located unidirectional origin, and fragments containing a stalled replication fork can produce a pattern with an inflection point indicative of a termination site.[60] The neutral-alkaline gel method readily reveals the direction of fork travel through a given DNA segment,[56] but it has the drawback that any nicking of parental strands during DNA isolation can obscure the diagonal display of nascent DNA. Furthermore, it is difficult to detect the end of the diagonal that corresponds to the shortest nascent fragment length that is used to determine the position of the origin. Finally, 2-D gel hybridization patterns in higher eukaryotes appear more complex than would be expected from theory. These difficulties tend to increase with the genome complexity, and may involve other types of DNA rearrangements such as branch migration at replication forks.[22]

IV. METHODS FOR IDENTIFICATION OF ORIGINS BASED ON FUNCTIONALITY

Functional assays for replication origins have been reviewed recently for their application to yeast.[24,65] Their applicability to higher eukaryotes remains questionable.[11,90] If cis-acting sequences are required for origin function in cellular chromosomes, then any segment of DNA that contains these sequences would be expected to confer on plasmids the ability to replicate autonomously as extrachromosomal elements in homologous cells. This is the rationale that led to identification of plasmids containing autonomously replicating sequence (ARS) elements from yeast.[86,87]

A. ARS Assays in Yeast

The first and most widely used yeast ARS assay is the high-frequency transformation assay.[86,87] Plasmids containing a selectable yeast marker gene are used as vectors to clone DNA sequences of interest and then to transform yeast cells. Colonies of transformed cells are counted and the frequency of transformation for each plasmids is calculated. ARS-containing plasmids increase the efficiency of transformation of yeast cells by 100- to 10,000-fold. Because, on average, 25% of autonomously replicating plasmids are lost during each cell division,[17] ARS screening vectors must also include a yeast centromere. The centromere allows normal segregation and mitotic stability. Addition of a reporter gene such as β-galactosidase permits easier identification of the transformed yeast cells.[85] ARS-selection vectors are also used with genes whose expression alters cellular metabolism and produces a red pigment corresponding in intensity to the plasmid copy number.[50] Centromere-containing vectors have been used in two general selection schemes: (1) measurement of plasmid loss rates under relaxed selective pressure[17,36] and (2) measurement of the number of colonies with plasmids under full selective pressure (mitotic stability assay[64,86]).

High-frequency transformation assays permitted functional identification of the first eukaryotic chromosomal origin of replication, the ARS 1 sequence, and later of many other yeast ARS elements as well as ARS elements present in DNA from other organisms.[65] This assay also has been used in conjunction with mutational analysis for precise identification of the cis-acting sequence elements important for ARS function.[59]

1. Advantages

The high-frequency transformation assay is a fast, easy, and reliable test for identification of

cis-acting DNA sequences involved in initiation of replication in yeast cells.

2. Limitations

The high-frequency transformation assay is applicable only to yeast. It is limited in its ability to quantify ARS function because ARS activity and transformation efficiency are not quantitatively related. In addition, integration of plasmids, which is difficult to avoid, contributes to errors when the rate of ARS-plasmid loss is used as a quantitative measure. It also appears that, while all yeast chromosomal origins are ARS elements, not all ARS elements function as origins in the yeast chromosome.[24] Thus, ARS assays alone are not sufficient to identify chromosomal origins of DNA replication. Therefore, one of the other methods for mapping initiation sites on chromosomal DNA described earlier (e.g., 2-D gel method) must be applied on the genomic copy of each ARS element. Moreover, the ARS assay could be misleading by identifying as ARS elements, sequences that only mimic structurally the ARS consensus and other elements important for ARS function in yeast. This led in the early 1980s to the identification of a large number of sequences from different heterologous organisms active as ARS in yeast cells as origins of replication.[65] In fact, active ARS elements can be created within the vector sequences by selecting for mutations that support autonomous plasmid replication in yeast.[47]

B. ARS Assay in Mammalian Cells

1. Long-Term Assays

Specific plasmid vectors have been created from the Epstein-Barr virus (EBV) that allow isolation of ARS plasmids in mammalian cells.[51,100] EBV maintains a low copy number (10 to 100 copies per cell) in human cells. These plasmid vectors lack the dyad symmetry element of the EBV *oriP* that is required for the virus to replicate in human cells. However, the plasmids do contain the family of repeats that binds EBNA-1 as well as the EBNA-1 gene that provide a nuclear retention capability. They also contain a hygromycin resistance gene that allows for selection of transfected cells. These vectors have allowed isolation of human DNA sequences that function as mammalian ARS. Under hygromycin selection they are maintained as episomes for at least 2 months. Human DNA sequences are much more efficient than prokaryotic sequences at maintaining plasmid replication, and longer fragments are more efficient than smaller ones.[35] However, specific origins of replication have not yet been identified.[52]

Another long-term ARS assay relies entirely on selective pressure to maintain the plasmid in the cell nucleus.[62] For that purpose, a selectable gene is included in the vector such as the bacterial neomycin resistance gene. After several cell divisions in the presence of G418, replicationally incompetent plasmids are lost and, in the absence of neomycin gene product, those cells die. Only plasmids that both replicate and are transmitted from one cell generation to the next can confer drug resistance. Selective pressure can be applied to cells for an unlimited time. Plasmids carrying the neomycin resistance gene and a 2.5 kb DNA fragment upstream of the human c-*myc* gene have been maintained for more than 300 cell generations in HeLa cells under G418.

A third approach is based on the use of a specific plasmid vector containing a selectable marker gene, the herpes simplex virus thymidine kinase (tk) gene driven by a truncated promoter to reduce its activity.[39] Under these conditions, viral thymidine kinase is produced at subthreshold levels in the transfected tk^- cells, and these cells die when cultured in HAT medium. However, if one of the fragments of cellular DNA cloned into this vector carries a mammalian ARS element, then plasmid replication will lead to an increased number of plasmids per cell and the level of viral thymidine kinase will increase. These cells should survive in HAT medium. Using this approach, 19 putative mouse ARS containing DNA fragments were isolated.[39] However, it was

later found that all of these plasmids had integrated into cellular chromosomes as tandem repeats.[27]

2. Short-Term Assays

In mammalian cells, plasmids containing a putative ARS elements are transfected or injected into cell nuclei by a variety of techniques, and their ability to replicate assayed 24 to 96 h later using one or both of the following approaches.

a. Dpn I/MboI Assay

Plasmids are grown in bacteria containing deoxyadenosine methylase (dam^+) in order to methylate Dpn I restriction sites and make them sensitive to cleavage by Dpn I. However, since mammalian cells lack this methylase, these plasmids become hemimethylated and then nonmethylated if they undergo one or more rounds of replication in a mammalian cell. This results in resistance to Dpn I (Figure 10). Mbo I has the same recognition sequence as Dpn I, but Mbo I cleaves only unmethylated DNA. Thus, by comparing results of Dpn I digestion with those from Mbo I digestion, one round of plasmid DNA replication (hemimethylated) can be distinguished from two or more rounds of replication (nonmethylated) in mammalian cells (Figure 10).

Plasmids carrying a putative mammalian origin are used to transfect cultured mammalian cells by one of several available techniques.[78] Most of these plasmids lack sequences that may inhibit plasmid replication in mammalian cells.[58] The vector itself should be cotransfected with the test plasmid in order to provide an "ori$^-$" internal standard. Transfected cells are incubated for 24 to 96 h before plasmid DNA is recovered, usually by the procedure of Hirt.[38] The purified DNA is then cut at a single restriction site in order to convert all replication products into linear DNA molecules of unit length. This serves to collect all Dpn I resistant plasmid molecules into a single DNA band that is more easily detected following gel electrophoresis. DNA is then digested with either Dpn I or Mbo I. Dpn I digestion converts unreplicated DNA to smaller fragments, while Mbo I converts replicated DNA to smaller fragments. DNA is then fractionated by gel electrophoresis and detected by blotting-hybridization using appropriate ^{32}P-DNA probes.

The optimal conditions for digestion of replicated plasmid DNA with Dpn I has been rein-

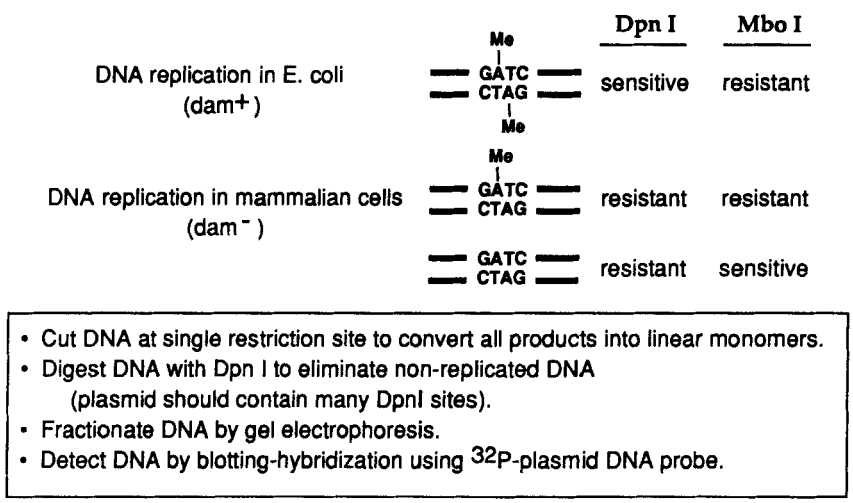

FIGURE 10. Principle of the Dpn I/Mbo I assay for plasmid DNA replication in mammalian cells. (Adapted from Peden et al., 1980.)

vestigated recently.[79] Maximum enzyme activity is found at salt concentrations <50 m*M* NaCl, but Dpn I can also digest a portion of the hemimethylated DNA. At salt concentration >150 m*M* NaCl, Dpn I does not cleave hemimethylated DNA, but the enzyme also does not digest unmethylated DNA to completion. Thus, the optimal salt concentration is 100 m*M*. Addition of the polycation spermidine appears to be essential for complete digestion of plasmids recovered from mammalian cells.[6,61,79]

Short-term Dpn I resistance ARS assays have been developed and used widely in studying cis-acting sequence elements important for origin function in animal viruses.[11] They have also been applied to studying putative mammalian chromosomal origins of replication.[25,43,62]

An alternative method for detecting Dpn I resistant plasmid DNA following transfection of mammalian cells is to measure its ability to reestablish itself as a plasmid in bacterial cells.[91] If the putative ARS element is cloned into an ampicillin resistant pBR vector, it can be cotransfected into competent bacterial cells together with a control vector that is both ampicillin and tetracyclin resistant. Following transformation, bacteria are plated onto agar containing both antibiotics. The number of bacterial colonies that form are proportional to the number of plasmid molecules recovered from mammalian cells that survived Dpn I digestion. A modification of this approach uses pUC vectors.[76] These simplify colony counting by producing blue (vector alone) and white (vector plus recombinant DNA) colonies on the same plate. Using highly transformation-competent cells, one can detect levels of Dpn I-resistant plasmids that are not easily detectable by blotting-hybridization.

b. Density Substitution Assay

One round of semiconservative replication in the presence of BrdU produces plasmid DNA containing one light strand with Thd and one heavy strand substituted with BrdU. Multiple rounds of replication produce DNA molecules with two heavy strands. Transfected eukaryotic cells are grown in the presence of BrdU to density-label replicating plasmids. Plasmids are recovered and partially purified from cellular DNA by the method of Hirt.[38] They are usually linearized by appropriate restriction enzymes and fractionated according to their density by centrifugation to equilibrium in CsCl gradients. Plasmid sequences are detected by blotting-hybridization using vector-specific ^{32}P-labeled probes. This technique has been used to characterize putative mammalian origins,[25] as well as DNA replication in a large number of plasmid and viral DNA molecules.

c. Advantages

ARS assays are the simplest and most direct approach to identifying cis-acting DNA sequences that can function as origins of replication in metazoan organisms. A large number of plasmids carrying putative replication origins or deletion mutants can be tested in a single experiment. Retransformation of bacteria provides an easy method for quantitation, as well as accurate internal control for completion of Dpn I digestion, because retransformation measures primarily form I plasmid DNA. It has been reported that Dpn I resistant form I is a more accurate assessment of replication than Dpn I-resistant linear DNA.[70] The most important advantage of ARS assays is their ability to identify eukaryotic origins that function at low efficiency.

d. Limitations

Although used successfully in studying replication origins of animal viruses, ARS assays have generated mixed results in detecting sequence-specific origin activity in metazoan organisms. The pros and cons of various reports of ARS elements that function in mammalian cells are discussed by Umek et al.[90] and Burhans et al.[11] Theoretically, the ability of a given DNA sequence to support autonomous replication should reflect origin function. In practice, however, with selective pressure one can expect that sequences inactive as origins in the chromosomes can be forced to act as origins on plasmids.

V. CHOOSING AN APPROPRIATE METHOD

One dark night, a policeman found a man walking around under a street lamp searching for his lost keys. When asked where he thought he had lost them, the man pointed across the street. If you lost them over there, asked the policeman, then why are you looking for them here? Because this is where the light is, the man replied. As this anecdote suggests, there are basically two approaches to the identification and isolation of origins of DNA replication: one can look where there is already light or one can search the unexplored regions of the genome.

Many regions of eukaryotic DNA have already been cloned and partially sequenced. These regions may or may not contain origins of replication. In such cases, the choice of mapping techniques depends on several parameters:

- Number of copies per genome
- Size of the genomic locus
- Replication time during S-phase
- Ability to synchronize cells
- Ability of the cell to take up DNA synthesis precursors
- Availability of sequence information

If one is studying lower eukaryotic cells, such as yeast or *Physarum,* that contain a small genome and do not allow efficient uptake of DNA precursors, then the most suitable methods are 2-D gel electrophoresis or the kinetic approach for measuring nascent DNA strand length. If one is studying higher eukaryotic cells, such as those from mammals whose genome is at least 200-fold more complex than yeast and whose DNA can be readily labeled with a variety of DNA synthesis precursors, then we suggest analysis of newly synthesized DNA based on nascent DNA strand length or replication fork polarity. The matrix enrichment variant of the 2-D gel electrophoresis method is also applicable. However, in one experimental system (the DHFR region in CHO cells), the 2-D gel electrophoresis method[22] gave results that appear to be in contradiction to results from five different methods for identification of origins based on analysis of newly synthesized DNA strands (earliest labeled DNA fragment, replication origin trap, PCR approach to measuring nascent DNA strand length, Okazaki fragment distribution, and imbalanced DNA synthesis methods). If one is studying a single-copy sequence rather than a naturally occurring multicopy sequence (e.g., rDNA genes), and if one does not have available cell lines in which the single-copy sequence has been amplified (e.g., DHFR region in CHO C400 cells), then one is limited to the nascent DNA strand length, replication fork polarity, and 2-D gel electrophoresis methods.

When >10 kb of sequence is to be searched for origins, replication fork polarity methods allow one to identify forward (leading) and retrograde (lagging) template strands at a single site, and thus reveal on which side of this site the origin is located. Of the two replication fork polarity approaches, the imbalanced DNA synthesis approach is the simpler technique for estimating where an origin of bidirectional replication is located and the outer limits of its replication unit. However, the resolution of this technique is limited. If the origin is activated at the onset of S-phase, then one can apply the Okazaki fragment distribution approach. This method provides higher resolution and stronger evidence for the existence of an origin of bidirectional replication, although it is technically more laborious. If one is not sure when their sequence of interest is replicated, a relatively simple method for determining time of replication during S-phase has been developed recently.[81] If the origin is not activated at the onset of S-phase, then one should apply the PCR approach for measuring nascent strand length. Of all the available methods, only two are capable of examining single-copy sequences without cell synchronization or treatment of cells with metabolic inhibitors: the PCR approach for measuring nascent strand lengths, and 2-D gel electrophoresis.

How does one search for origins in the unexplored regions of the genome? Each mammalian cell contains tens of thousands of replication origins that might differ in their structure and regulation. Therefore, analysis of a single replication origin may fall short of understanding how most replication origins function. Here we

suggest a general strategy for identification and mapping of unknown replication origins from the same organism.

Instead of walking along hundreds of kilobases of DNA in search of an origin, one can apply an origin selection method. The replication origin trap and nascent DNA strand extrusion methods produce a library of origin-enriched sequences. Unique clones from this library can be used as probes to isolate the corresponding genomic copies in which the origins reside in their natural chromosomal environment. Initiation sites can then be localized as described previously. Alternatively, functional assays for origins can be applied in the hope of finding an ARS sequence.

The following specific examples serve to illustrate strategies for origin identification.

Example 1: Assume circumstantial evidence suggests that a replication initiation site is located within several kilobases upstream of a known single-copy mammalian gene. Assume ~10 kb of sequence flanking the gene is also available, but that its replication timing is unknown. The most appropriate method to apply is the PCR approach of the nascent DNA strand method.

Example 2: Assume DNA clones and partial sequence information is available for a 100-kb single copy mammalian DNA locus replicating at the beginning of the S-phase. Assume there is no evidence for initiation of replication within this locus, but probability suggests that at least one origin will be found (average origin to origin distance is 30 to 300 kb). One approach is to use either of the replication fork polarity methods to first establish the direction of fork travel at one DNA site. Then one can spot-check DNA segments every ~10 kb or so in order to flank the nearest origin of bidirectional replication.

Example 3: Assume an origin of replication is located somewhere within each of the 200 tandem copies of mammalian rDNA, probably in the nontranscribed spacer region, as suggested for rDNA in other organisms. The repeated unit is approximately 50 kb and DNA clones are available for most of its length. The high copy number of the repeated locus permits most of the techniques described in this review to be applied. However, in our experience, the Okazaki fragment distribution method is most likely to identify the origin with greatest precision.

Example 4: Assume an origin of replication has been mapped in a single copy mammalian chromosomal DNA locus that does not replicate at the beginning of S-phase. The objective is to identify all neighboring origins and the organization of the whole cluster of replication units. For this purpose at least several kilobases of DNA on both sides of the origin should be mapped and clones should be made that at least partially cover the locus. The only practical technique for mapping the boundaries of replication units and the approximate location of their origins is the imbalanced DNA synthesis method. After some of the origins in the locus are located within several kilobases of DNA, additional sequence information can be obtained and the initiation site can be mapped more precisely using the PCR approach of the nascent DNA strand length method.

VI. SOME FINAL THOUGHTS

Even with this new arsenal of methods, identification of replication origins in eukaryotic chromosomes is not a trivial task. While ARS elements are now well established as origins in yeast chromosomes, site-specific origins of bidirectional DNA replication have only been identified recently in higher eukaryotes, and the question remains as to their relationship with specific cis-acting sequences such as those found in the chromosomes of bacteria, yeast, and animal viruses.[11] It is likely that initiation of replication in animal cells that require one and only one duplication of their genes each cell cycle is regulated differently than in simpler cells and viruses. Therefore, at present one cannot be certain whether the initiation zones identified by the various methods described above actually occupy several kilobases of sequence or simply reflect either low resolution or some artifact in the mapping technique. The answer will require a thoughtful, critical application of more than one method to the same system.

ACKNOWLEDGMENTS

We are indebted to Dr. William Burhans (Roswell Park Cancer Institute, Buffalo, NY), Dr. Craig Chinault (Baylor College of Medicine,

Houston, TX), and Dr. Geoff Wahl (Salk Institute, La Jolla, CA) for their constructive comments and for sharing unpublished results with us.

REFERENCES

1. **Anachkova, B. and Hamlin, J. L.,** Replication in the amplified dihydrofolate reductase domain in CHO cells may initiate at two distinct sites, one of which is a repetitive sequence element, *Mol. Cell. Biol.*, 9, 532, 1989.
2. **Bell, L. and Byers, B.,** Occurrence of crossed strand-exchange forms in yeast DNA during meiosis, *Proc. Natl. Acad. Sci. U.S.A.*, 76, 3445, 1981.
3. **Benard, M. and Pierron, G.,** Physical relationship between a gene and its origin of replication in *Physarum polycephalum, Exp. Cell. Res.*, 186, 299, 1990.
4. **Blumenthal, A. B., Kriegstein, H. J., and Hogness, D. S.,** The units of DNA replication in *Drosophila melanogaster* chromosomes, *Cold Spring Harbor Symp. Quant. Biol.*, 38, 205, 1974.
5. **Botchan, P. M. and Dayton, A. I.,** A specific replication origin in the chromosomal rDNA of *Lytechinus variagatus, Nature*, 299, 453, 1982.
6. **Bouche, J. P.,** The effect of spermidine on endonuclease inhibition by agarose contaminants, *Anal. Biochem.*, 115, 42, 1981.
7. **Bozzoni, I., Baldari, C. T., Amaldi, F., and Buongiorno-Nardelli, D.,** Replication of ribosomal DNA in *Xenopus leavis, Eur. J. Biochem.*, 118, 585, 1981.
8. **Brewer, B. J. and Fangman, W. L.,** The localization of replication origins on ARS plasmids in *S. cerevisiae, Cell*, 51, 463, 1987.
9. **Brewer, B. J. and Fangman, W. L.,** A replicational fork barrier at the 3′ end of yeast ribosomal RNA genes, *Cell*, 55, 637, 1988.
10. **Burhans, W. C., Vassilev, L. T., Wu, J., Sogo, J. M., Nallaseth, F., and DePamphilis, M. L.,** Emetine allows identification of origins of mammalian DNA replication by imbalanced DNA synthesis, not through conservative nucleosome segregation, *EMBO J.*, 10, 4351, 1991.
11. **Burhans, W. C., Ortega, J. M., and DePamphilis, M. L.,** Eukaryotic origins of replication: what are they and how do they work?, *Crit. Rev. Biochem. Mol. Biol.*, submitted.
12. **Burhans, W. C., Vassilev, L. T., Caddle, M. S., Heintz, N. H., and DePamphilis, M. L.,** Identification of an origin of bidirectional DNA replication in mammalian chromosomes, *Cell*, 62, 955, 1990.
13. **Burhans, W. C., Selegue, J. E., and Heintz, N. H.,** Isolation of the origin of replication associated with the amplified CHO dehydrofolatereductase domain, *Proc. Natl. Acad. Sci. U.S.A.*, 83, 7790, 1986.
14. **Carroll, S. M., Gaudray, P., DeRose, M. L., Emery, J. F., Meinkoth, J. L., Nakkim, E., Subler, M., VonHoff, D. D., and Wahl, G. M.,** Characterization of an episome produced in hamster cells that amplify a transfected CAD gene at high frequency: functional evidence for a mammalian replication origin, *Mol. Cell. Biol.*, 7, 1740, 1987.
15. **Cech, T. R. and Brehm, S. L.,** Replication of the extrachromosomal ribosomal RNA genes of *Tetrahymena thermophilia, Nucl. Acids Res.*, 9, 3531, 1981.

15a. **Contreas, G., Giacca, M., and Falaschi, A.,** Purification of BrdUrd-substituted DNA by immunoaffinity chromatography with anti-BrdUrd antibodies, *BioTechniques*, 12, 824, 1992.

16. **Cusick, M. E., DePamphilis, M. L., and Wassarman, P. M.,** Dispersive segregation of nucleosomes during replication of Simian Virus 40 chromosomes, *J. Mol. Biol.*, 178, 249, 1984.
17. **Dani, G. and Zakian, V.,** Mitotic and meiotic stability of linear plasmids in yeast, *Proc. Natl. Acad. Sci. U.S.A.*, 80, 3406, 1983.
18. **Delidakis, C. and Kafatos, F. C.,** Amplification enhancers and replication origins in the autosomal chorion gene cluster of *Drosophila, EMBO J.*, 8, 891, 1989.
19. **DePamphilis, M. L.,** Replication of simian virus 40 and polyoma virus chromosomes, *Molecular Aspects of Papovaviruses*, Aloni, Y., Ed., Martinus Nijhoff, Boston, 1987, 1.
20. **DePamphilis, M. L. and Wassarman, P. M.,** Organization and replication of papovavirus DNA, in *Organization and Replication of Viral DNA*, Kaplan, A. S., Ed., CRC Press, Boca Raton, 1982, 37.
21. **DePamphilis, M. L. and Wassarman, P. M.,** Replication of eukaryotic chromosomes: a close-up of the replication fork, *Ann. Rev. Biochem.*, 49, 627, 1980.
22. **Dijkwel, P. A., Vaughn, J. P., and Hamlin, J. L.,** 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.*, 11, 3850, 1991.
23. **Edenberg, H. J. and Huberman, J. A.,** Eukaryotic chromosome replication, *Annu. Rev. Genet.*, 9, 245, 1975.
24. **Fangman, W. L. and Brewer, B. J.,** Activation of replication origins within yeast chromosomes, *Ann. Rev. Cell Biol.*, 7, 375, 1991.
25. **Frappier, L. and Zannis-Hadjopoulos, M.,** Autonomous replication of plasmids bearing monkey DNA origin-enriched sequences, *Proc. Natl. Acad. Sci. U.S.A.*, 84, 6668, 1987.
26. **Gann, T. A. and Schildkraut, C. L.,** The Epstein-Barr virus origin of plasmid replication, ori P, contains both the initiation and termination sites of DNA replication, *Cell*, 58, 527, 1989.

27. **Gilbert, D. and Cohen, S. N.,** Autonomous replication in mouse cells: a correction, *Cell,* 56, 1433.
28. **Guo, Z.-S., Heine, U., and DePamphilis, M. L.,** T-antigen binding to site I facilitates initiation of SV40 DNA replication but does not affect bidirectionality, *Nucl. Acids Res.*, 19, 7081, 1991.
29. **Hand, R.,** Eukaryotic chromosome replication and its regulation, in *Cell Biology, A Comprehensive Treatise,* Vol. 2, Prescott, D. M. and Goldstein, L., Eds., Academic Press, Orlando, 1979, 389.
30. **Handeli, S., Klar, A., Meuth, M., and Cedar, H.,** Mapping replication units in animal cells, *Cell,* 57, 909, 1989.
31. **Hay, R. T. and DePamphilis, M. L.,** Initiation of simian virus 40 DNA replication *in vivo:* location and structure of 5′-ends of DNA synthesized in the *ori*-region, *Cell,* 28, 767, 1982.
32. **Heck, M. M. S. and Spradling, A. C.,** Multiple replication origins are used during *Drosophila* chorion gene amplification, *J. Cell Biol.*, 110, 903, 1990.
33. **Heintz, N. H. and Hamlin, J. L.,** An amplified chromosomal sequence that includes the gene for dehydrofolate reductase initiate replication within specific restriction fragment, *Proc. Natl. Acad. Sci. U.S.A.*, 79, 4083, 1982.
34. **Heintz, N. H. and Stillman, B. W.,** Nuclear DNA synthesis *in vitro* is mediated via stable replication forks assembled in a temporally specific fashion *in vivo, Mol. Cell. Biol.*, 8, 1923, 1988.
35. **Heinzel, S. S., Krysan, P. J., Tran, C. T., and Calos, M. P.,** Autonomous DNA replication in human cells is affected by the size and source of the DNA, *Mol. Cell. Biol.*, 11, 2263, 1991.
36. **Heiter, P., Mann, C., Snyder, M., and Davis, R.,** Mitotic stability of yeast chromosomes: a colony color assay that measures nondisjunction and chromosome loss, *Cell,* 381, 1985.
37. **Hendrickson, E. A., Fritze, C. E., Folk, W. R., and DePamphilis, M. L.,** The origin of bidirectional DNA replication in Polyoma virus, *EMBO J.*, 6, 2011, 1987.
38. **Hirt, B.,** Selective extraction of polyoma DNA from infected mouse cell cultures, *J. Mol. Biol.*, 26, 365, 1967.
39. **Holst, A., Muler, F., Zastrow, G., Zentgraf, F., Schwender, S., Dinkl, E., and Grummt, F.,** Murine genomic DNA sequences replicating autonomously in mouse L cells, *Cell,* 52, 355, 1988.
40. **Huberman, J. A., Spotila, L. D., Nowotka, K. A., El-Assouli, S. M., and Davis, L. R.,** The *in vivo* replication origin of the yeast 2 μm plasmid, *Cell,* 51, 473, 1987.
41. **Huberman, J. and Riggs, A.,** On the mechanism of DNA replication in mammalian chromosomes, *J. Mol. Biol.*, 32, 327, 1968.
42. **Huberman, J., Zhu, J., Davis, L., and Newlon, C.,** Close association of a DNA replication origin and an ARS element on chromosome III of the yeast *Saccharomyces cerevisiae, Nucl. Acids Res.*, 16, 6373, 1988.
42a. **Hyrien, O. and Méchali, M.,** Plasmid replication in *Xenopus* eggs and egg extracts: a 2-D gel electrophoretic analysis, *Nucl. Acids Res.*, 20, 1463, 1992.
43. **Igushi-Ariga, S., Okazaki, T., Itani, T., Ogata, M., Sato, Y., and Ariga, H.,** An initiation site of DNA replication with transcriptional enhancer activity present upstream of the c-*myc* gene, *EMBO J.*, 7, 3135, 1988.
44. **Jacob, F., Brenner, J., and Cuzin, F.,** On the regulation of DNA replication in bacteria, *Cold Spring Harbor Symp. Quant. Biol.*, 28, 329, 1963.
45. **James, C. D. and Leffak, M.,** Polarity of DNA replication through the avian alpha-globin locus, *Mol. Cell. Biol.*, 6, 976, 1986.
46. **Kaufmann, G., Zannis-Hadjopoulos, M., and Martin, R. G.,** Cloning of nascent monkey DNA synthesized early in the cell cycle, *Mol. Cell. Biol.*, 5, 721, 1985.
47. **Kipling, D. and Kearsey, S. E.,** Reversion of autonomously replicating sequence mutations in *Saccharomyces cerevisiae.* Creation of a eukaryotic replication origin within procaryotic vector DNA, *Mol. Cell. Biol.*, 10, 265, 1990.
48. **Kohara, Y., Tohdoh, N., Jiang, X.-W., and Okazaki, T.,** The distribution and properties of RNA primed initiation sites of DNA synthesis at the replication origin of *Escherichia coli* chromosome, *Nucl. Acids Res.*, 13, 6847, 1985.
49. **Kornberg, A. and Baker, T.,** *DNA replication,* 2nd Ed., Freeman and Company, New York, 1992.
50. **Koshland, D., Kent, J., and Hartwell, L.,** Genetic analysis of the mitotic transmission of minichromosomes, *Cell,* 40, 393, 1985.
51. **Krysan, P., Haase, S., and Calos, M.,** Isolation of human sequences that replicate autonomously in human cells, *Mol. Cell. Biol.*, 9, 1026, 1989.
52. **Krysan, P. J. and Calos, M. P.,** Replication initiates at multiple locations on an autonomously replicating plasmid in human cells, *Mol. Cell. Biol.*, 11, 1464, 1991.
53. **Laskey, R. A.,** The use of intensifying screens or organic scintillators for visualizing radioactive molecules resolved by gel electrophoresis, *Meth. Enzymology,* 65, 363, 1980.
54. **Leu, T.-H. and Hamlin, J. L.,** High-resolution mapping of replication fork movement through the amplified dehydrofolate reductase domain in CHO cells by in-gel renaturation analysis, *Mol. Cell. Biol.*, 9, 523, 1989.
55. **Linskens, M. H. K. and Huberman, J. A.,** The two faces of higher eukaryotic DNA replication origins, *Cell,* 62, 845, 1990.
56. **Linskens, M. H. K. and Huberman, J. A.,** Organization of replication of ribosomal DNA in *Saccharomyces cerevisiae, Mol. Cell. Biol.*, 8, 4927, 1988.

57. **Linskens, M. H. K. and Huberman, J. A.,** Ambiguities in results obtained with 2-D gel replicon mapping techniques, *Nucl. Acids Res.*, 18, 647, 1990.
58. **Lusky, M. and Botchan, M.,** Inhibition of SV40 replication in simian cells by specific pBR322 DNA sequences, *Nature*, 293, 79, 1981.
59. **Marahrens, Y. and Stillman, B.,** A yeast chromosomal origin of DNA replication defined by multiple functional elements, *Science*, 255, 817, 1992.
60. **Martin-Parras, L., Hernández, P., Martínez-Robles, M. L., and Schvartzman, J. B.,** Unidirectional replication as visualized by two-dimensional agarose gel electrophoresis, *J. Mol. Biol.*, 220, 843, 1991.
61. **Martínez-Salas, E., Cupo, D. Y., and DePamphilis, M. L.,** The need for enhancers is acquired upon formation of a diploid nucleus during early mouse development, *Genes Develop.*, 2, 1115, 1988.
62. **McWhinney, C. and Leffak, M.,** Autonomous replication of a DNA fragment containing the chromosomal replication origin of the human c-*myc* gene, *Nucl. Acids Res.*, 18, 1233, 1990.
63. **Micheli, G., Baldari, C. T., Carri, M. T., Cello, G. D., and Buongiorno-Nardelli, M.,** An electron microscope study of chromosomal DNA replication in different eukaryotic systems, *Exp. Cell. Res.*, 137, 127, 1982.
64. **Murray, A. W. and Szostak, J. W.,** Pedigree analysis of plasmid segregation in yeast, *Cell*, 34, 961, 1983.
65. **Newlon, C. S.,** Yeast chromosome replication and segregation, *Microbiol. Rev.*, 52, 568, 1988.
66. **Nowotka, K. A. and Huberman, J. A.,** Two-dimensional electrophoretic method for mapping DNA replicons, *Mol. Cell. Biol.*, 8, 1408, 1988.
67. **Peden, K. W., Pipas, J. M., Pearson-White, S., and Nathans, D.,** Isolation of mutants of an animal virus in bacteria, *Science*, 209, 1392, 1980.
68. **Petes, T. D. and Newlon, C. S.,** Structure of DNA in DNA replication mutants of yeast, *Nature*, 251, 637, 1974.
69. **Prescott, D. and Kuempel, P.,** Autoradiography of individual DNA molecules, *Methods Cell Biol.*, 7, 147, 1973.
70. **Rao, B. S. and Martin, R. G.,** Dpn I assay for DNA replication in animal cells: enzyme resistant material can result from factors not related to replication, *Nucl. Acids Res.*, 16, 4171, 1988.
71. **Razin, S. V., Kekelidze, M. G., Lukanidin, E. M., Scherrer, K., and Georgiev, G. P.,** Replication origins are attached to the nuclear skeleton, *Nucl. Acids Res.*, 14, 8189, 1986.
72. **Riley, D. and Weintraub, H.,** Conservative segregation of parental histones during replication in the presence of cycloheximide, *Proc. Natl. Acad. Sci. U.S.A.*, 76, 328, 1979.
73. **Robinson, I.,** Detection and mapping of homologous, repeated and amplified DNA sequences by DNA renaturation in agarose gels, *Nucl. Acids Res.*, 11, 5413, 1983.
74. **Rokeach, L. A. and Zyskind, J. W.,** RNA terminating within the *E. coli* origin of replication: stringent regulation and control by dnaA protein, *Cell*, 46, 763, 1986.
75. **Roufa, D. J. and Marchionni, M. A.,** Nucleosome segregation at a defined mammalian chromosomal site, *Proc. Natl. Acad. Sci. U.S.A.*, 79, 1810, 1982.
76. **Rusconi, S., Severne, Y., Georgiev, O., Galli, I., and Wieland, S.,** A novel expression assay to study transcriptional activators, *Gene*, 89, 211, 1990.
77. **Russev, G. and Vassilev, L.,** Isolation of a DNA fraction from Ehrlich ascites tumour cells containing the putative origin of replication, *J. Mol. Biol.*, 161, 77, 1982.
78. **Sambrook, J., Fritcsh, E., and Maniatis, T.,** *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989.
79. **Sanchez, J. A., Marek, D., and Wangh, L. J.,** Proof of efficient semiconservative plasmid replication in *Xenopus* eggs using an optimized "SENSE" assay, submitted.
80. **Seidman, M. M., Levine, A. J., and Weintraub, H.,** The asymmetric segregation of parental nucleosomes during chromosome replication, *Cell*, 18, 439, 1979.
81. **Selig, S., Okumura, K., Ward, D. C., and Cedar, H.,** Delineation of DNA replication time zones by fluorescence in *in situ* hybridization, *EMBO J.*, 11, 1217, 1992.
82. **Seufert, W. and Messer, W.,** Start sites for bidirectional *in vitro* DNA replication inside the replication origin, oriC, of *Escherichia coli*, *EMBO J.*, 6, 2469, 1987.
83. **Shinomiya, T. and Ina, S.,** Analysis of chromosomal replicons in early embryos of Drosophila melanogaster by two-dimensional gel electrophoresis, *Nucl. Acids Res.*, 19, 3935, 1991.
84. **Sogo, J. M., Stahl, H., Koller, Th., and Knippers, R.,** Structure of replicating SV40 minichromosomes: the replication fork, core histone segregation and terminal structures, *J. Mol. Biol.*, 189, 189, 1986.
85. **Srienc, F., Bailey, J., and Campbell, J.,** Effect of ARS 1 mutations on chromosome stability in *Saccharomyces cerevisiae*, *Mol. Cell. Biol.*, 5, 1676, 1985.
86. **Stinchcomb, D. T., Struhl, K., and Davis, R. W.,** Isolation and characterization of a yeast chromosomal replicator, *Nature*, 282, 39, 1979.
87. **Struhl, K., Stinchkomb, D., Scherer, S., and Davis, R.,** High-frequency transformation of yeast: autonomous replication of hybrid DNA molecules, *Proc. Natl. Acad. Sci. U.S.A.*, 76, 1035, 1979.
88. **Truett, M. A. and Gall, J. G.,** The replication of the ribosomal DNA in the macronucleus of Tetrahymena, *Chromosoma*, 64, 295, 1977.

89. **Tseng, B. Y. and Goulian, M.,** DNA synthesis in human lymphocytes: intermediates in DNA synthesis, *in vitro* and *in vivo*, *J. Mol. Biol.*, 99, 317, 1975.
90. **Umek, R. M., Linskens, M. H. K., Kowalski, D., and Huberman, J. A.,** New beginnings in studies of eukaryotic DNA replication origins, *Biochim. Biophys. Acta*, 1007, 1, 1989.
91. **Vassilev, L. and Johnson, E. M.,** Evaluation of autonomous plasmid replication in transfected mammalian cells, *Nucl. Acids Res.*, 16, 7742, 1988.
92. **Vassilev, L. and Johnson, E. M.,** Mapping initiation sites of DNA replication *in vivo* using polymerase chain reaction amplification of nascent strand segments, *Nucl. Acids Res.*, 19, 7693, 1989.
93. **Vassilev, L. and Johnson, E. M.,** An initiation zone of chromosomal DNA replication located upstream of the c-*myc* gene in proliferating HeLa cells, *Mol. Cell. Biol.*, 10, 4899, 1990.
94. **Vassilev, L. and Russev, G.,** Purification of nascent DNA chains by immunoprecipitation with anti-BrdU antibodies, *Nucl. Acids Res.*, 16, 10397, 1988.
95. **Vassilev, L. T., Burhans, W. C., and DePamphilis, M. L.,** Mapping an origin of DNA replication at a single copy locus in exponentially proliferating mammalian cells, *Mol. Cell. Biol.*, 10, 4685, 1990.
96. **Vaughn, J. P., Dijkwel, P. A., and Hamlin, J. L.,** Replication initiates in a broad zone in the amplified CHO dihydrofolate reductase domain, *Cell*, 61, 1075, 1990.
97. **Vaughn, J. P., Dijkwel, P. A., and Hamlin, J. L.,** Initiation of DNA replication occurs in a broad zone in the DHFR gene locus, *Cell*, 61, 1075, 1990.
98. **Vogt, V. M. and Braun, R.,** The replication of ribosomal DNA in *Physarum polycephalum*, *Eur. J. Biochem.*, 80, 557, 1977.
99. **Yang, L. and Botchan, M.,** Replication of bovine papillomavirus type 1 DNA initiates within an E2 responsive enhancer element, *J. Virol.*, 64, 5903, 1990.
100. **Yates, J. L. and Guan, N.,** Epstein-Barr virus-derived plasmids replicate only once per cell cycle and are not amplified after entry into cells, *J. Virol.*, 65, 483, 1991.
101. **Yoda, K.-Y., Yasuda, H., Jiang, X.-W., and Okazaki, T.,** RNA-primed initiation sites of DNA replication in the origin region of bacteriophage λ genome, *Nucl. Acids Res.*, 16, 6531, 1988.
102. **Zannis-Hadjopoulos, M., Persico, M., and Martin, R. G.,** The remarkable instability of replication loops provides a general method for the isolation of origins of DNA replication, *Cell*, 27, 155, 1981.