Site-specific Initiation of DNA Replication in Metazoan Chromosomes and the Role of Nuclear Organization

D.M. GILBERT,* H. MIYAZAWA,* F.S. NALLASETH,* J.M. ORTEGA,*
J.J. BLOW,† AND M.L. DEPAMPHILIS*

*Roche Institute of Molecular Biology, Roche Research Center, Nutley, New Jersey 07110;
†ICRF Clare Hall Laboratories, South Mimms, Potters Bar, Herts EN63LD, United Kingdom

Analyses of DNA replication in "simple" genomes that replicate in eukaryotic cells such as animal viruses and mitochondria as well as the chromosomes in protozoa (*Tetrahymena*), yeast (*Saccharomyces*), and slime mold (*Physarum*) have led to the generally held view that replication begins when specific proteins bind specific DNA sequences to initiate DNA unwinding at or near the binding site (for review, see Kornberg and Baker 1992; DePamphilis 1993a). This event is followed rapidly by initiation of DNA synthesis on one or both DNA templates. DNA replication in cellular chromosomes of differentiated cells as well as in circular DNA molecules that replicate in their nuclei, such as papovaviruses and papillomaviruses, all utilize the standard replication fork mechanism outlined in Figure 1 (for review, see Burhans et al. 1990). DNA is synthesized continuously on the forward arm (leading strand) but discontinuously on the retrograde arm (lagging strand) by the repeated synthesis and joining of short RNA-primed, nascent DNA chains (Fig. 1, Okazaki fragments). Okazaki fragments themselves may be assembled from a cluster of even shorter RNA-DNA chains of <40 nucleotides (Nethanel et al. 1992). One consequence of the asymmetrical distribution of Okazaki fragments at replication forks is that an origin of bidirectional replication (OBR) can be identified by the transition from discontinuous to continuous DNA synthesis that must occur on each template.

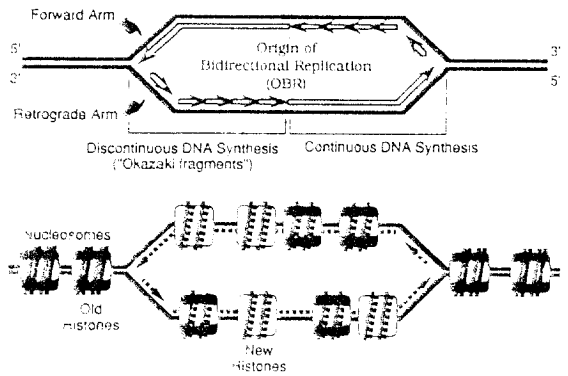

Figure 1. DNA structure at origins of bidirectional replication, and the near-random distribution of new and old histone octamers between both arms of replication forks.

In SV40 and polyomaviruses, this transition occurs within 2 bp (Hay and DePamphilis 1982) and 20 bp (Hendrickson et al. 1987), respectively. As the replication fork passes through a nucleosome, the old histone octamer in front of the fork is distributed randomly to either arm (Fig. 1) (Burhans et al. 1991; Krude and Knippers 1991; Sugasawa et al. 1992), and new histone octamers are rapidly assembled by a two-step reaction in which histones H3/H4 are deposited first, followed by histones H2A/H2B (Gruss and Sogo 1992). As two forks approach one another, topoisomerase II is required for unwinding the final region of duplex DNA separating the forks (Ishimi et al. 1992). Termination of replication does not require specific sequences (Zhu et al. 1992), but specific sequences can impede DNA unwinding in this termination region, resulting in formation of catenated intertwines that also require topoisomerase II for their resolution (Fields-Berry and DePamphilis 1989).

In simple genomes, the genetic origin (*ori*), defined by *cis*-acting mutations, and the functional origin, defined as the actual site where replication begins (e.g., the OBR), are coincident and encompass from 50 bp to ~1000 bp of DNA (Kornberg and Baker 1992; DePamphilis 1993a). Most of these origins have been shown to act as *a*utonomously *r*eplicating *s*equences (ARS) by conferring on other DNA molecules the ability to replicate when transferred to either cells or cell extracts containing the required replication proteins. Simple origins consist of two basic components: a core component that is the minimal essential *cis*-acting sequence required to initiate DNA replication under all conditions, and one or more auxiliary components that affect the efficiency but not the mechanism of replication. The core component is analogous to a transcription promoter, whereas the auxiliary components, which are dispensable under some conditions, are analogous to (sometimes identical to) transcription enhancers. The core component is the binding site for an origin recognition protein (e.g., SV40 T antigen) and includes the site where DNA unwinding begins, whereas auxiliary components consist of DNA-binding sites for transcription factors that increase *ori*-core activity from 2- to 1000-fold, depending on experimental conditions (DePamphilis 1989, 1993c). Thus, simple "eu-

karyotic" origins appear similar in design to origins of replication in prokaryotic cells, plasmids, and bacteriophage (Kornberg and Baker 1992).

Metazoan Chromosomes Initiate Replication at Specific Sites

In contrast to simple origins, origins of replication in the chromosomes of metazoan animals appear more complex (for review, see DePamphilis 1993a,b). During the past few years, several methods have been developed for mapping sites in metazoan chromosomes where replication begins (for summary, see Vassilev and DePamphilis 1992). These methods can be divided into two groups: The first group identifies the origin of newly synthesized DNA by incorporation of labeled deoxyribonucleotides. The second group detects DNA structures resembling replication forks and bubbles.

The first group of methods includes identifying the earliest labeled DNA fragment, trapping replication bubbles at the site of their origin by cross-linking DNA with psoralen, measuring the lengths of unique nascent DNA strands in order to locate the OBR at their center, and identifying replication fork polarity. Fork polarity can be established by hybridization of either Okazaki fragments or nascent leading strand DNA to each DNA template. Nascent leading strands can be isolated when Okazaki fragment synthesis is preferentially inhibited in the presence of protein synthesis inhibitors such as emetine or cycloheximide, because the resulting imbalanced DNA synthesis at replication forks allows leading strand synthesis to continue in the absence of lagging strand synthesis. By examining fork polarity at several chromosomal locations, one can identify the transition from continuous to discontinuous DNA synthesis that defines an OBR (Fig. 1). The first OBR identified in metazoan chromosomes (OBR-1) is located ~17 kb downstream from the hamster dihydrofolate reductase (DHFR) gene and corresponds to the earliest labeled DNA fragment in this region, *oriβ* (Table 1). Other origins that have been identified include a late S-phase origin 160 kb downstream from the mouse adenosine deaminase (ADA) gene, at the 5′ end of the human c-myc gene, within the transcribed region of an undefined human gene (cDNA 343), within the mouse CAD gene, at the 5′ end of the hamster rhodopsin gene, between the human δ- and β-globin genes, and an early S-phase OBR 29 kb upstream of the mouse ADA gene (Table 1). The best-characterized OBR, the DHFR OBR-1, is confined to a region as small as 0.5 kb but not larger than 2 kb (Table 1). Several other OBRs are also located within a 2-kb segment, and still others have been mapped to regions of 4 kb to 11 kb. These regions indicate the outer limits of the OBR. Both the distribution of Okazaki fragments and the distribution of leading strands are consistent with 80–85% of replication forks outside the OBR traveling in one direction, suggesting that at least 80% of initiation events occur within these OBR regions.

Table 1. DNA Replication Begins at Specific Sites in Metazoan Chromosomes

Origins	Species	Genetic assays[a]	Mapping method[b]	OBR[c]	Initiation zone[d]	Reference
S-Phase origins						
DHFR gene (OBR-1)	hamster	translocation (ARS[+])	1–7	0.5–2 kb	55 kb	Handeli et al. (1989) Burhans et al. (1990, 1991) Vassilev et al. (1990) Dijkwel and Hamlin (1992)
rRNA genes	human		6, 7		31 kb	Little et al. (1993)
ADA gene (late S phase)	mouse	ARS episome[e]	3	2 kb		Virta-Pearlman et al. (1993)
c-*myc* gene	human	ARS	1, 3, 4	2 kb		McWhinney and Leffak (1990) M. Leffak (unpubl.) Vassilev and Johnson (1990)
cDNA 343	human	ARS	3	2 kb		Wu et al. (1993)
CAD gene	mouse	episome[e]	1, 4, 5	4 kb		G. Wahl (unpubl.)
rhodopsin gene	hamster		1, 3	5 kb		Gale et al. (1992)
β-globin gene	human	deletion	5	5 kb		D. Kitsberg and H. Cedar (unpubl.)
ADA gene (early S phase)	mouse	episome[e]	1, 4	11 kb		Carroll et al. (1993)
Amplification origins						
chorion gene	*Drosophila*	translocation	6, 7	1 kb	8 kb	Delidakis and Kafatos (1989) Heck and Spradling (1990)
locus 9, chrom-2	*Sciara*		6, 7	1 kb	9 kb	Liang et al. (1993)

[a] Translocation to another chromosomal site reproduced OBR. ARS supports autonomous replication in plasmids; episome is extrachromosomal element containing multiple tandem copies of the gene; deletion of OBR region in chromosome eliminated origin activity.

[b] Methods are (1) early labeled DNA fragment, (2) origin trap, (3) nascent DNA strand length, (4) Okazaki fragment distribution, (5) imbalanced DNA synthesis, (6) neutral-neutral 2-D gel replication bubbles, (7) neutral-alkaline 2-D gel replication forks.

[c] OBRs in S-phase origins were identified by analysis of newly synthesized DNA; OBRs in amplification origins were defined by 2-D gel analysis of replication fork direction.

[d] Initiation zones were defined by 2-D gel analysis of replication bubbles.

[e] The term episome is used here to imply extrachromosomal genetic element, not as originally intended definition (Wollman et al. 1957) as an element that naturally exists in both integrated and extrachromosomal forms.

The second group of methods fractionates total DNA by 2-D gel electrophoresis and then identifies the presence of replication bubbles and replication forks by their mobility patterns under various conditions. The genomic map locations of these structures are determined by hybridization with sequence-specific probes. Neutral-neutral 2-D gel electrophoresis is frequently used to identify the presence of replication bubbles. Neutral-alkaline 2-D gel electrophoresis is frequently used to identify replication fork polarity relative to a specific restriction enzyme cleavage site. In contrast to the nascent DNA strand methods described above, 2-D gel methods detect replication forks and bubbles distributed equally throughout a region that varies from 6 kb to 55 kb, depending on the origin examined (Table 1). These replication bubbles define an "initiation zone" within which is located the OBR. 2-D gel electrophoretic analyses of replication fork polarity at the *Drosophila* and *Sciara* developmentally programed amplification origins reveal that 80% and 90% of replication forks, respectively, originate from a 1-kb site (OBR) located within a 6-kb to 8-kb initiation zone. Similarly, OBR-1 is centrally located within the hamster DHFR 55-kb initiation zone. Thus, most initiation events at either a developmentally programed DNA amplification origin or an S-phase origin occur within a 0.5-kb to 2-kb OBR located within a larger initiation zone defined by the presence of additional replication bubbles.

Mapping DNA replication initiation sites in metazoan chromosomes reveals two important conclusions. First, DNA replication in the chromosomes of differentiated cells occurs by the same replication fork mechanism used in simple genomes. This was shown by the asymmetric distribution of Okazaki fragments between the two DNA templates, by the opposite asymmetric distribution in leading strands when imbalanced DNA synthesis was induced, and by the mobility patterns generated by replicating DNA structures during 2-D gel electrophoresis. The second conclusion is that initiation of DNA replication does not occur randomly throughout the entire genome but is localized to specific sites. Analyses of newly synthesized DNA strands at S-phase origins and of replication fork structures at amplified origins using 2-D gel electrophoresis reveal that at least 80% of initiation events originate bidirectionally from a specific site (the OBR) on the order of 0.5 kb to 2 kb in size. Although replication bubbles are often detected outside the OBR using 2-D gel electrophoresis, the resulting initiation zone is still limited in size and, in the case of the DHFR initiation region and the human rDNA locus, is confined to the nontranscribed intergenic region.

Perhaps the most puzzling aspect of metazoan origins of DNA replication has been the difficulty in identifying them by genetic analysis using many of the same techniques that have proven successful with simple genomes. Three of the origins mapped in chromosomes have been reported to exhibit ARS activity in mammalian cells, but these results have not yet been confirmed by other laboratories, and no mammalian ARS activity has been observed for the DHFR OBR-1 region (Table 1). Moreover, in some plasmid assays, any mammalian DNA sequence greater than 10 kb appears to support autonomous DNA replication (Caddle and Calos 1992), and virtually any DNA can replicate when injected into frog eggs or incubated in frog egg extracts (Hyrien and Méchali 1992; Mahbubani et al. 1992). Initiation occurs in these systems, on average, at one randomly chosen site in each DNA molecule. Consistent with these observations is the presence of replication bubbles (detected by 2-D gel electrophoresis) randomly distributed throughout the tandem repeats of histone genes in early *Drosophila* embryos (Shinomiya and Ina 1991), as well as observations that the frequency of initiation events in cleavage stage embryos of amphibians and flies is much greater than in differentiated cells of the same animal (Blumenthal et al. 1974; McKnight and Miller 1977; Buongiorno-Nardelli et al. 1982). Such results suggest that initiation of replication in metazoa is not tightly coupled to specific DNA sequences and that the availablity of origins may change during animal development.

On the other hand, the evidence is compelling that OBR sites are genetically determined. First, the simple fact that origins can be mapped at specific sites in vivo demands that origins are inherited from one cell division to the next, and the well-documented temporal order of replication (Gilbert 1986; Hatton et al. 1988 and references therein) would not be possible if replication initiated at random sites. Moreover, the same OBR identified in cells containing a single copy of the chromosomal region is also identified in cells containing multiple copies at either amplified chromosomal or extrachromosomal regions (DHFR OBR-1, ADA gene, and CAD gene origins). Therefore, the information that determines these OBRs must be encoded in the DNA sequence that is amplified. Evidence in support of this conclusion comes from reports that the DHFR OBR-1 (Handeli et al. 1989) and chorion gene amplification (Orr-Weaver 1991) origins retain their activity when translocated to other chromosomal sites. Conversely, the naturally occurring hemoglobin Lepore deletion, which encompasses the only chromosomal OBR found within a 135-kb region around the human β-globin gene, eliminates OBR activity in this region (D. Kitsberg and H. Cedar, unpubl.). These data demonstrate that metazoan origins of replication are determined by as yet undefined DNA sequences. For this reason, we suggest the terminology origin of bidirectional replication (OBR) to designate a functionally defined initiation site, reserving the term "ori" to designate the as yet undefined DNA sequences that comprise an origin.

How can one account for the varied and sometimes contradictory observations on DNA replication in metazoan nuclei? The simplest model is suggested by the Jesuit dictum that "many are called, but few are chosen." Although naked DNA appears to contain

many possible sites where replication can begin, assembly of DNA into chromatin followed by organization of the chromatin into a nuclear structure may repress some origin sites while activating others (Fig. 2) (DePamphilis 1993a,b). This appears to be the situation in yeast, where many ARS elements that function as origins in plasmids do not function as origins in chromosomes (see Newlon et al., this volume). Thus, in vivo, most newly synthesized DNA would originate from a small locus (the OBR), although initiation events may still occur at a low frequency throughout a larger initiation zone because of the presence of potential origin sequences. From this point of view, results of 2-D gel analyses are consistent with newly synthesized DNA analyses if one assumes that the fraction of initiation events that occur at the OBR is much greater than the fraction of initiation events that occur in any similar-sized region outside the OBR. In practice, the fraction of replication bubbles observed in any one DNA segment is difficult to determine by 2-D gel analysis (Dijkwel and Hamlin 1992; Little et al. 1993), whereas hybridization of Okazaki fragments or nascent leading strand DNA to separated DNA templates provides a quantitative assessment of fork polarity. Nascent DNA generated by replication bubbles randomly distributed outside the OBR would simply contribute to the background in origin-mapping methods that focus on radiolabeled nascent DNA.

The amount of DNA that is accessible to the DNA replication machinery may define the boundaries of an initiation zone. Whereas some initiation zones are confined to intergenic regions (DHFR, rRNA genes), some OBRs have been reported to reside within a structural gene (cDNA 343, CAD gene). Therefore, although initiation events are always restricted to specific sites, they are not necessarily restricted to intergenic regions. One advantage of an origin of replication that consists of specific sequences organized into a nucleoprotein structure is that replication would disassemble this structure and thus inactivate the origin. This would limit initiation events at each origin to one and only one per S phase, an essential characteristic of chromosome replication in metazoan cells.

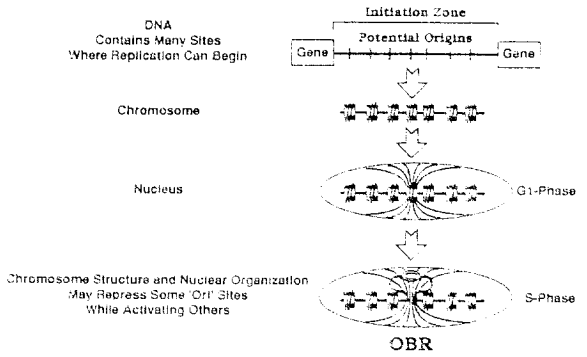

Figure 2. "Jesuit model" of origins of replication in metazoan chromosomes.

The necessity for nuclear organization in metazoan DNA replication is based primarily on the fact that replication in *Xenopus* eggs does not occur unless DNA is first assembled into chromatin and then organized into a nuclear structure that includes lamin III (Blow and Sleeman 1990; Newport et al. 1990; Meier et al. 1991). Chromatin structure can suppress access to alternative origins (Forrester et al. 1990; Simpson 1990; Karpen and Spradling 1990; Ferguson and Fangman 1992), whereas nuclear structure may be required to promote DNA unwinding (Bode et al. 1992). In addition, the nuclear envelope is instrumental in regulating the onset of S phase, apparently by regulating access of chromosomal DNA to one or more initiation factors ("licensing factor") present in the cytoplasm (Blow and Laskey 1988; Leno et al. 1992). The question remains as to whether nuclear organization also is involved in determining site-specific initiation of DNA replication in metazoan chromosomes.

RESULTS AND DISCUSSION

Site-specific Initiation of DNA Replication in *Xenopus* Egg Extracts Requires Nuclear Organization

In an effort to test the Jesuit model, we have searched for conditions under which DNA replication could be initiated in a subcellular system at the same DNA sites utilized by mammalian cells in vivo. To this end, we examined the ability of *Xenopus* eggs to initiate DNA replication at the hamster DHFR OBR-1. *Xenopus* eggs and egg extracts have been shown to carry out the sequential pathway outlined in Figure 2. They first assemble naked DNA into chromatin and then assemble chromatin into a nuclear structure. These steps are prerequisites for initiation of DNA replication. Replication is semiconservative and limited to one round per cell cycle (Blow and Laskey 1986). However, replication also appears not to require specific origin sequences.

We first determined whether or not with naked DNA as a substrate, *Xenopus* eggs would prefer an 11-kb sequence containing the DHFR OBR (OBR-1) mapped in hamster cells (pneoS13, kindly provided by J. Hamlin) to an equivalent-sized region taken either from the DHFR gene itself, which lies outside the initiation zone, or from bacteriophage λ DNA, which is presumably devoid of eukaryotic origins. These three segments were cloned into plasmids and coinjected either into fertilized *Xenopus* eggs that then developed to the blastula stage or into unfertilized eggs that were then allowed to convert the injected DNA into chromatin before activating the eggs in the presence of Ca^{++} and a Ca^{++} ionophore to initiate DNA replication (Wangh 1989). Plasmid DNA was purified, linearized, and then digested with either *Dpn*I to eliminate all DNA that had not undergone at least one round of DNA replication, or *Mbo*I to eliminate all DNA that had undergone at least two rounds of DNA replication.

The DNA products were fractionated by gel electrophoresis and identified by blotting hybridization using appropriate ^{32}P-labeled probes. All three plasmids replicated efficiently and to the same extent: no preference was observed for any of the three sequences. The same results were obtained when linear rather than circular plasmid DNA was injected, and when the amount of injected DNA was varied.

To allow better manipulation of experimental conditions, extracts of unfertilized *Xenopus* eggs were prepared according to the method of Blow and Laskey (1986). Both circular and linear plasmids were incubated in these extracts under conditions previously reported to initiate DNA replication in plasmids or sperm chromatin. Temperature and ratio of DNA to egg protein also were varied. In addition, histone H1, which is absent from chromatin assembled in *Xenopus* eggs (Laskey et al. 1985), was added at varying concentrations in an effort to create a chromatin structure analogous to that found in mammalian somatic cells. Under all conditions, all three plasmids replicated at the same rate and to the same extent. Thus, neither *Xenopus* eggs nor egg extracts revealed any preference for a known mammalian OBR when presented to the replication machinery as either naked DNA or DNA assembled into chromatin by the egg itself.

The next question was whether or not preformed nuclei isolated from hamster cells would initiate replication in *Xenopus* egg extract, and, if so, would these extracts preferentially initiate DNA synthesis at the same chromosomal sites chosen by the hamster cell. Therefore, CHOC 400 cells, containing ~1000 tandemly arranged copies of a 273-kb sequence containing the DHFR gene region (Heintz and Hamlin 1982), were synchronized by mitotic shake-off, and nuclei were isolated 4 hours after mitosis (G_1-phase nuclei) and 12 hours after mitosis (S-phase nuclei). Under these conditions, less than 1% of G_1 cells and greater than 70% of S-phase cells incorporate nucleotide precursors during a 30-minute labeling period in vivo at the time of collection. In addition, lysolecithin-treated, demembranized sperm nuclei (Gurdon 1976) were included as a control. These bundles of sperm chromatin have previously been shown to undergo DNA replication in *Xenopus* egg extracts that are capable of restoring their nuclear membrane (Blow and Laskey 1986).

All three substrates were first tested in extracts from S-phase Chinese hamster ovary (CHO) cells as described by Heintz and Stillman (1988). S-phase CHOC 400 nuclei commenced incorporation of radiolabeled deoxyribonucleotides immediately, but no DNA synthesis was detected either in G_1-phase CHOC 400 nuclei or in sperm chromatin (Fig. 3). This was consistent with previous reports that extracts from S-phase mammalian cells can support DNA replication at sites that already initiated replication in vivo but cannot initiate replication de novo (Heintz and Stillman 1988). When the same substrates were incubated in an extract from unfertilized activated *Xenopus* eggs made as described by Blow and Laskey (1986), S-phase nuclei again commenced DNA synthesis immediately, but, in contrast to the CHO cell extract, both G_1-phase nuclei and sperm chromatin initiated DNA synthesis after a characteristic lag period (Fig. 3). The same result also was observed when either nuclei or chromatin was incubated with BrdUTP and then stained with an antibody directed against this nucleotide. Thus, G_1-phase nuclei as well as sperm chromatin are easily distinguished from S-phase nuclei in three ways: (1) Less than 1% of the G_1 cell populations used to prepare G_1 nuclei have begun to synthesize DNA in vivo, whereas 70% of S-phase nuclei were synthesizing DNA. (2) Extracts from S-phase hamster cells support DNA synthesis in S-phase nuclei, but not in G_1-phase nuclei or sperm chromatin and (3) extracts from *Xenopus* eggs support DNA synthesis in all three substrates, but G_1-phase nuclei and sperm chromatin require a preparatory period before beginning DNA synthesis. The preparatory period observed with G_1-phase nuclei did not appear to result from a requirement for de novo nuclear membrane formation, because *Xenopus* egg extracts that had either been centrifuged to remove their membrane vesicle fraction or diluted ~50% still initiated DNA replication in G_1

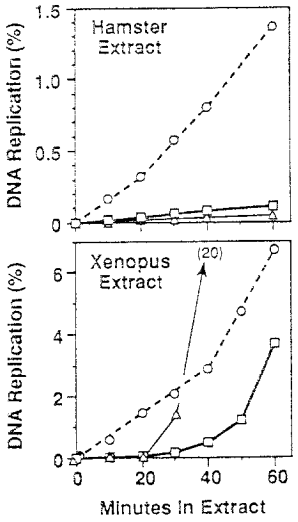

Figure 3. Distinguishing nuclei from G_1-phase and S-phase CHO cells on the basis of their abilities to replicate in different cell extracts. The CHO cell line CHOC 400 was synchronized by mitotic selection and plated to fresh media for 4 hr (G_1) or 12 hr (S phase). BrdU labeling for 30 min prior to collection revealed that 0.2% of G_1 nuclei and 70% of S-phase nuclei were synthesizing DNA in vivo. G_1 nuclei (□), S-phase nuclei (○), and *Xenopus* sperm chromatin (△) were tested for their ability to replicate their DNA in a cytosolic extract from S-phase CHO cells (Hamster Extract) or an extract from unfertilized activated *Xenopus* eggs (Xenopus Extract). DNA replication was measured as incorporation of [^{32}P]dATP into acid-soluble DNA and then nascent ^{32}P-labeled DNA expressed as a fraction of the total DNA substrate. Nuclei (prepared from CHOC 400 cells) and S-phase cytosol (prepared from CHO K1 cells) were prepared as described by Heintz and Stillman (1988). *Xenopus* egg extracts and sperm chromatin were prepared as described by Blow and Laskey (1986).

nuclei as described in Figure 3, but failed to initiate DNA synthesis in sperm chromatin. Therefore, preformed S-phase and G_1-phase hamster nuclei may be responding to soluble DNA replication factors present in *Xenopus* egg extracts. However, it is possible that any tears or holes in the G_1 nuclei are repaired by residual membrane vesicles in the extract during this preparatory period.

DNA synthesis observed in hamster G_1-phase nuclei incubated in *Xenopus* egg extract represented semiconservative DNA replication rather than DNA repair. This was demonstrated in two ways. First, the newly synthesized DNA was labeled with BrdUTP as well as [^{32}P]dATP and fractionated according to its density in Cs_2SO_4 gradients at neutral pH. Newly synthesized ^{32}P-labeled DNA comigrated with a heavy-light DNA standard, consistent with semiconservative DNA replication. This conclusion was confirmed by isolating the heavy-light ^{32}P-labeled DNA, denaturing it in Cs_2SO_4 at alkaline pH, and again fractionating the DNA according to its density. At least 90% of the ^{32}P-labeled DNA from the heavy-light hybrid then comigrated with the heavy strand from the DNA standard, demonstrating that all of the newly synthesized DNA resided in the same strand. Second, DNA synthesis in G_1-phase nuclei was sensitive to aphidicolin or araCTP and resistant to ddTTP, consistent with DNA synthesis being carried out by DNA polymerases-α and -δ, but not by DNA polymerase-β.

The next question was whether or not DNA replication in S-phase and G_1-phase nuclei in *Xenopus* extracts occurred at the same sites used by the hamster cell. The simplest method to begin looking for origins of replication in cell chromosomes is the early labeled fragment assay. This was the first method that revealed the presence of a specific origin of replication in mammalian chromosomes (DHFR *ori*β; Heintz and Hamlin 1982), a conclusion that was subsequently confirmed both by improvements in identifying early labeled DNA fragments and by several other assays such as measuring the lengths of unique nascent DNA strands and identification of replication fork polarity either by Okazaki fragment distribution or by imbalanced DNA synthesis (for review, see DePamphilis 1993a,b).

The resolution of the early labeled fragment assay was first evaluated using nuclei from hamster CHOC 400 cells that had been synchronized by mitotic shake-off and then arrested at their G_1/S-phase boundary with aphidicolin, a specific inhibitor of DNA polymerases-α and -δ. These nuclei have initiated DNA replication in vivo and have begun to synthesize short nascent DNA strands, but they are prevented by the aphidicolin from elongating these nascent strands away from the OBR. These early S-phase nuclei were then released from their aphidicolin block and incubated for 20 minutes in an extract from S-phase CHO cells supplemented with [^{32}P]dATP to label nascent DNA in the vicinity of the OBR. DNA was then purified, digested with *Eco*RI, and fractionated by gel electrophoresis; ^{32}P-labeled DNA fragments were identified by autoradiography (Fig. 4). Because CHOC 400 cells contain ~1000 tandem copies of the DHFR gene region, restriction enzyme fragments from this region appear as sharp bands above a uniform background of ^{32}P-labeled DNA fragments from the remainder of the genome. Statistically, *Eco*RI should produce about 66 DNA fragments from the 273-kb amplified region. At least three origins have been identified in this region (DePamphilis 1993b), and others probably exist as well. OBR-1 is located within one of two adjacent 6-kb *Eco*RI fragments that comigrate during gel electrophoresis. Nuclei from unsynchronized, exponentially

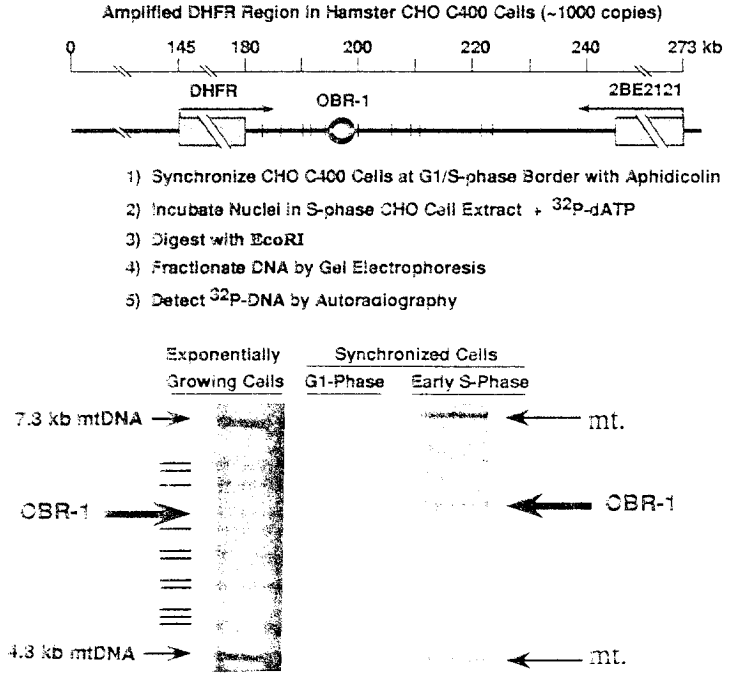

Figure 4. Early labeled fragment assay for detecting site-specific initiation of DNA replication. At the top is a schematic diagram of the amplified DHFR locus and the positions of a few of the mapped *Eco*RI sites, as well as the location of OBR-1. The procedure is as described by Heintz and Stillman (1988) except that CHOC 400 cells were synchronized by mitotic selection and collected either 4 hr after mitosis in complete medium (G$_1$-Phase) or 12 hr after mitosis in complete medium containing 5 μg/ml aphidicolin (Early S-Phase). The tick marks on the left of the autoradiogram indicate the positions of visible amplified bands migrating at positions between the 7.8-kb and 4.8-kb CHO mitochondrial bands; the heavy arrow marks the position of the OBR-1-containing restriction fragment.

growing cells generate a series of *Eco*RI ^{32}P-labeled DNA fragments that were similar in intensity: 12 of these bands migrate between two DNA fragments derived from mitochondrial DNA. Nuclei from G_1-phase CHOC 400 cells (isolated 4 hr prior to S phase) incorporated little or no [^{32}P]dATP, but nuclei that had just entered S phase produced a strongly labeled DNA band consistent with initiation of replication in the OBR-1 region. These data are in excellent agreement with those published previously by Heintz and Stillman (1988). Radiolabel that appears in other *Eco*RI DNA fragments reflects (1) the degree of asynchrony within this population of cells and (2) the fact that initiation events also occur at other origins within this 273-kb amplified region.

To test the ability of *Xenopus* egg extract to initiate DNA synthesis at the same sites chosen by the hamster cell, the experimental protocol was modified to compensate for the fact that G_1-phase nuclei did not initiate replication synchronously in vitro. Nuclei first were incubated in a *Xenopus* egg extract for 1 hour in the absence of [^{32}P]dATP and in the presence of aphidicolin. Nuclei were then washed free of aphidicolin and immediately incubated in a second extract from S-phase hamster CHO cells in the presence of [^{32}P]dATP. This allowed replication bubbles that were initiated in the first extract to incorporate radiolabel into their nascent DNA chains in the second extract. DNA was extracted after the second incubation had proceeded for 20, 40, or 60 minutes. As a control, half of the nuclei were incubated in an S-phase CHO cell extract during the first incubation, under the same conditions used for the *Xenopus* egg extract, and then transferred to fresh CHO cell extract for the second incubation. This control corrected for any effects the protocol may have had on our ability to detect site-specific initiation of DNA replication at OBR-1.

With nuclei that were incubated exclusively in CHO cell extracts, no ^{32}P-labeled DNA bands were detected with G_1-phase nuclei except those derived from mitochondrial DNA, whereas nuclei from aphidicolin-arrested cells (early S-phase nuclei) incorporated [^{32}P]dATP most rapidly into the OBR-1-containing DNA fragment (Fig. 5, "Hamster Cell Extract"). When early S-phase nuclei were incubated for 1 hour in a *Xenopus* egg extract and then allowed to undergo DNA synthesis in a hamster cell extract (Fig. 5, "Xenopus Egg Extract"), the pattern of ^{32}P-labeled DNA bands was strikingly similar to those observed in hamster extracts alone. Therefore, *Xenopus* egg extract did not erase the pattern of initiation events that had been started in hamster cells and allowed to continue in S-phase hamster cell extract. A very similar pattern of ^{32}P-labeled DNA bands also appeared when the *Xenopus* egg extract initiated replication in G_1-phase nuclei (Fig. 5). In contrast, nuclei from exponentially growing cells always gave the same pattern of bands with equal intensity. In view of the previous

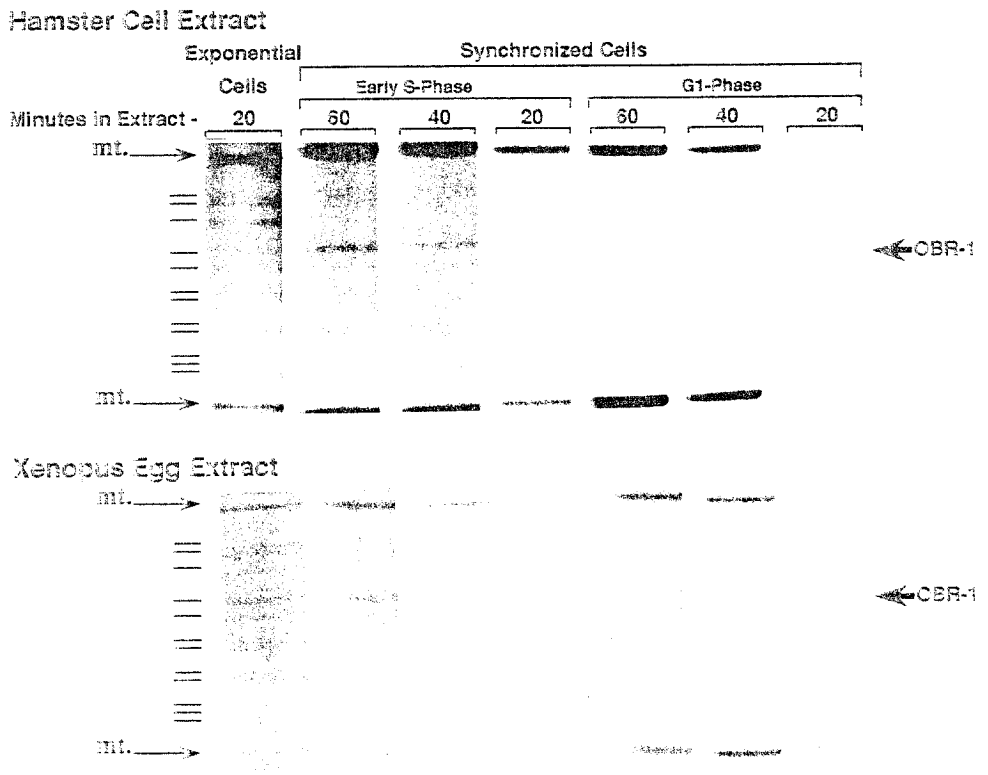

Figure 5. *Xenopus* egg extract initiates DNA replication in vitro at the same site normally chosen in vivo by the hamster cell. Nuclei were prepared as in Fig. 4, and equal numbers of each type of nucleus were incubated at 21°C in either *Xenopus* egg extract or CHO S-phase cytosol for 1 hr in the presence of aphidicolin. Nuclei were then washed free of the aphidicolin and incubated in CHO S-phase cytosol for the indicated times at 37°C. Band positions are as indicated in Fig. 4.

success of the early labeled DNA fragment assay in identifying origins of replication (Table 1), these data strongly suggest that *Xenopus* egg extract can preferentially initiate DNA replication in vitro at the same sites chosen in vivo by the hamster cell, but only when the DNA substrate is presented in the form of a nucleus. Experiments are now in progress to confirm and extend these results by identifying the transition between continuous and discontinuous DNA synthesis at OBR-1 based on the distribution of Okazaki fragments.

Papillomaviruses Are Not Suitable Models for Origins of Replication in Metazoan Chromosomes

Previous studies concluded that bovine papillomavirus (BPV) is a paradigm for cellular DNA replication because it appeared to replicate only once per S phase (Botchan et al. 1986), to maintain a low copy number per cell (Berg et al. 1986), and to contain two *cis*-acting negative control of replication (NCOR) sequences that, in the presence of the BPV M protein, suppress the lytic SV40 origin of replication so that it also maintains a low copy number (Roberts and Weintraub 1988). Subsequently, careful examination of BPV replication during the cell cycle revealed that BPV, like bacterial plasmids and the ribosomal DNA of *Physarum* (Vogt and Braun 1977), replicates randomly throughout S phase and does not restrict its replication to once per S phase (Gilbert and Cohen 1987; Gilbert 1989; Ravnan et al. 1992). Moreover, BPV DNA replication requires only the E1 and E2 proteins, and the BPV origin of DNA replication includes neither the *cis*-acting plasmid maintenance sequences nor the *cis*-acting NCOR sequences (for summary, see Ustav et al. 1993; Yang et al. 1993). Therefore, in an effort to further investigate the analogy between BPV and cellular DNA replication, DNA fragments from the BPV genome were examined for their ability to suppress the polyomavirus (PyV) origin of replication. Unlike SV40, PyV can replicate in the same mouse cells that replicate BPV, allowing analysis of both origins under identical conditions.

Our approach was to construct plasmids containing either the complete PyV origin alone (pPyV) or the complete PyV origin linked to various fragments of the BPV genome (Fig. 6, IM mutants). Each plasmid containing a BPV fragment was then mixed with pPyV and electroporated into mouse C127 cells, the usual laboratory host for BPV, along with an expression vector for PyV large tumor antigen (T-ag), the only PyV-encoded protein required to activate the PyV origin. These plasmids also were electroporated into FOP cells, mouse fibroblasts that express PyV T-ag from an integrated copy of the PyV T-ag gene. Our results confirmed that BPV contains two *cis*-acting sequences, corresponding to the NCOR-1 and NCOR-2 regions first identified by Roberts and Weintraub (1988), that can suppress PyV origin as well as SV40 origin activity. Results from both cell lines were equivalent except that levels of suppression in C127 cells were about 4-fold greater than in FOP cells. Suppression of PyV-driven plasmid DNA replication resulted from specific BPV sequences and not simply from changes in

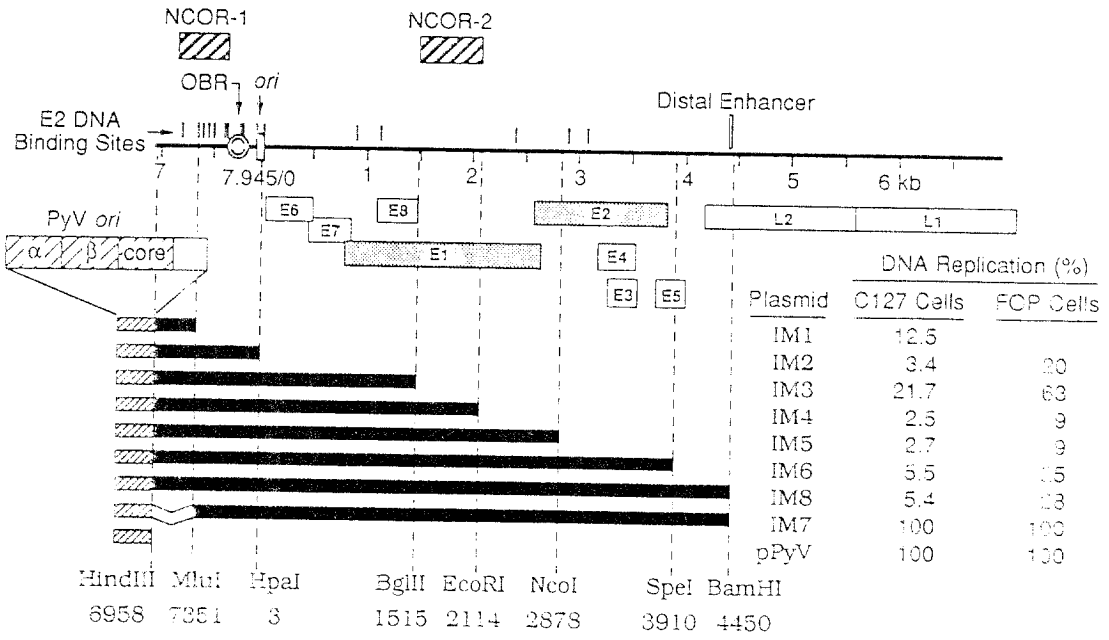

Figure 6. Identification of *cis*-acting NCOR sequences (*hatched areas*) in the BPV genome. The PyV origin of replication, consisting of the α, β, and core elements (DePamphilis et al. 1988) plus 50 additional base pairs of PyV DNA, was linked to the indicated DNA fragments from the BPV genome and inserted into pML-1. The DNA replication ability of each "insertion mutant" (IM) in either mouse C127 cells (PyV T-ag provided by a PyV T-ag gene expression vector) or FOP cells (PyV T-ag provided from integrated T-ag gene) is given relative to that of pPyV DNA present in the same cells. The locations of the BPV early (E) and late (L) genes are indicated, along with the genetically defined origin of replication (ori), the origin of bidirectional replication (OBR) defined by 2-D gel electrophoresis mapping of replication bubbles, and the locations of E2 DNA-binding sites.

plasmid size. The 5-kb BPV insert IM7 replicated as well as PyV alone, whereas larger (IM8) and smaller (IM2, IM4, IM5) inserts strongly suppressed PyV replication. With both NCOR-1 and NCOR-2 sequences present, PyV-driven origin activity was suppressed as much as 40-fold (Fig. 6, IM4 and IM5). Other BPV sequences (IM3, IM6) appeared to counter the effects of NCOR-1 and NCOR-2. However, neither NCOR-1 nor NCOR-2 appeared to be involved in regulating BPV DNA replication, because deletions of these sequences did not affect the efficiency of BPVori-driven replication in the absence of T-Ag.

About 75% of BPV's suppression of the PyV origin was localized to a 513-bp sequence, NCOR-1 (Fig. 6), whose activity was independent of distance and orientation with respect to the PyV origin. NCOR-1 did not encompass the BPV origin of replication, and suppression of the PyV origin by NCOR-1 did not require any BPV proteins. For example, suppression by IM2, which lacked any BPV genes, was equivalent to IM8, which contained all the BPV early genes. Moreover, coelectroporation of IM3 or IM8 with expression vectors for BPV E1 and E2 genes, or with the complete BPV genome in C127 cells, or electroporation of IM3 and IM8 into mouse cells transformed with BPV, had little or no effect on PyV origin activity in these mutants. In contrast, the extent of suppression was dependent on the concentration of PyV T-ag, suggesting that NCOR-1 interacted with this protein.

To determine whether or not NCOR-1 and NCOR-2 restricted PyV DNA replication to a single S phase, IM2 and IM4 were coelectroporated with pPyV and a PyV T-ag expression vector into mouse C127 cells in the presence of nocodazole. Nocodazole is a specific inhibitor of microtubule assembly that arrests cells in mitosis, thus preventing cells from undergoing more than one S phase. Plasmid DNA was extracted at various times after electroporation and then cut either with *Dpn*I restriction endonuclease to determine the fraction of DNA that underwent at least one round of replication or with *Mbo*I to determine the fraction of DNA that underwent at least two rounds of replication. The rate at which both IM2 and IM4 DNA became insensitive to *Dpn*I and then sensitive to *Mbo*I was indistinguishable from that of pPyV DNA. Therefore, although NCOR-1 alone and NCOR-1 plus NCOR-2 suppressed PyV origin activity, they did not do so by limiting the PyV origin to one initiation event per S phase. Instead, these BPV sequences appear to reduce the fraction of PyV origins that enter the replication cycle.

To determine whether or not NCOR-1 was involved in BPV DNA replication, IM7 and IM8 were coelectroporated into C127 cells with pML-1 (*dam*$^-$) and expression vectors for BPV E1 and E2 gene products. Under these conditions, plasmid DNA replication was dependent on the BPV origin of replication. The pML-1 was propagated in a *dam*$^-$ cell line to make it resistant to cleavage by *Dpn*I restriction endonuclease and thus provide an internal standard with which to compare the replication rates of different IM plasmids. The same experiment was also carried out with IM7 and IM8 constructions that lacked the PyV origin. Under these conditions, an expression vector for PyV T-ag could also be included to test the effects of PyV T-ag on NCOR activity. In the absence of PyV T-ag, and in either the presence or absence of the PyV origin, no difference was observed between replication of IM8, which contains the complete origin and early gene region of BPV, and IM7, which contains all of the same sequences except for NCOR-1. Therefore, NCOR-1 did not affect BPV DNA replication in transient assays. However, in the presence of T-ag (and the absence of the PyV origin), IM7 produced three times more DNA than did IM8. This result, together with the previously mentioned dependence of IM suppression of PyV origin activity on T-ag concentration, strongly suggests that suppression of the lytic PyV and SV40 origins by NCOR sequences results from interaction between these sequences and papovavirus T-ag. In the absence of T-ag, there is no evidence that NCOR sequences regulate BPV DNA replication. In fact, Chittenden et al. (1991) recently reported that SV40 origin-driven plasmids could establish an extrachromosomal state without any BPV sequences present. Therefore, it appears that NCOR sequences are not involved either in BPV DNA replication or in BPV copy number control, but simply reduce the efficiency of papovavirus replication through a spurious negative interaction with T-Ag.

Recent developments suggest that several of the results leading to the conclusion that BPV replication is regulated like chromosomal DNA replication are incorrect. Yang et al. (1991) point out that the basic experiments defining BPV plasmid maintenance sequences (Lusky and Botchan 1984) "cannot be repeated and are likely to be artifacts." The original experiment reported by Botchan et al. (1986), indicating that BPV is replicated once per cell cycle, has not been reproduced and was probably the result of integrated forms of BPV that came under control of flanking chromosomal sequences (Gilbert 1989; Ravnan et al. 1992; K. Ten-Hagen et al., in prep.). In fact, recent data (Ravnan et al. 1992) substantiate the original experiments of Gilbert and Cohen (1987) demonstrating that BPV replicates by a random choice mechanism with no temporal specificity. Furthermore, both late-passage and fresh subclones of BPV-transformed cell lines exhibit extreme cell to cell variability in the numbers of BPV molecules, with most cells having lost all BPV DNA (Ravnan et al. 1992; K. Ten-Hagen et al., in prep.), demonstrating that the copy number of BPV is not stably maintained. Considering these results and the results described in this paper, we conclude that BPV is a slow-replicating version of SV40 and PyV and not an appropriate model for cellular origins of replication.

SUMMARY

We have asked whether or not *Xenopus* eggs or egg extracts, which have previously been shown to replicate essentially any DNA molecule, will preferentially utilize a known mammalian OBR. Our results reveal that

Xenopus egg extracts can preferentially initiate DNA replication at sites chosen in vivo by the hamster cell, provided that the DNA substrate is presented to the extract in the form of a nucleus rather than bare DNA. Thus, site-specific initiation of DNA replication in metazoan cell chromosomes appears to be determined by nuclear organization as well as DNA sequence. We have also considered whether or not BPV, which was previously reported to regulate its copy number through negative as well as positive *cis*-acting sequences, provides a suitable paradigm for cellular origins. The BPV genome was found to contain *cis*-acting sequences that can suppress DNA replication driven by a lytic virus such as PyV. However, this suppression did not require any BPV protein, did not limit PyV origin activity to one initiation event per S phase, and did not affect BPV origin activity. These results, together with data from other laboratories, strongly suggest that BPV is simply a slow-replicating version of SV40 and PyV and therefore is not an appropriate model to explain how initiation of cellular DNA replication is limited to once per cell cycle.

ACKNOWLEDGMENTS

We extend our thanks to Julian Blow for his generous help in getting our laboratory started with the *Xenopus* egg system. J.J. Blow is a Lister Institute Centenary Fellow. H. Miyazawa is supported by a grant for "Biodesign Research Program" from RIKEN (Japan).

REFERENCES

Berg, L., M. Lusky, A. Stenlund, and M.R. Botchan. 1986. Repression of bovine papilloma virus replication is mediated by a virally encoded *trans*-acting factor. *Cell* **46:** 753.

Blow, J.J. and R.A. Laskey. 1986. Initiation of DNA replication in nuclei and purified DNA by a cell-free extract of *Xenopus* eggs. *Cell* **47:** 577.

———. 1988. A role for the nuclear envelope in controlling DNA replication within the cell cycle. *Nature* **332:** 546.

Blow, J.J. and A.M. Sleeman. 1990. Replication of purified DNA in *Xenopus* egg extracts is dependent on nuclear assembly. *J. Cell Sci.* **95:** 383.

Blumenthal, A.B., H.J. Kreigstein, and D.S. Hogness. 1974. The units of DNA replication in *Drosophila melanogaster* chromosomes. *Cold Spring Harbor Symp. Quant. Biol.* **38:** 205.

Bode, J., Y. Kohwi, L. Dickinson, T. Joh, D. Klehr, C. Mielke, and T. Kohwi-Shigematsu. 1992. Biological significance of unwinding capability of nuclear matrix-associating DNAs. *Science* **55:** 195.

Botchan, M.R., L.J. Berg, J. Reynolds, and M. Lusky. 1986. The bovine papilloma virus replicon. In *Papillomaviruses* (ed. D. Evered and S. Clark), p. 53. John Wiley, New York.

Buongiorno-Nardelli, M., G. Micheli, M.T. Carri, and M. Marilley. 1982. A relationship between replicon size and supercoiled loop domains in the eukaryotic genome. *Nature* **298:** 100.

Burhans, W.C., L.T. Vassilev, M.S. Caddle, N.H. Heintz, and M.L. DePamphilis. 1990. Identification of an origin of bidirectional DNA replication in mammalian chromosomes. *Cell* **62:** 955.

Burhans, W.C., L.T. Vassilev, J. Wu, J.M. Sogo, F.N. Nallaseth, and M.L. DePamphilis. 1991. Emetine allows identification of origins of mammalian DNA replication by imbalanced DNA synthesis, not through conservative nucleosome segregation. *EMBO J.* **10:** 4351.

Caddle, M.S. and M.P. Calos. 1992. Analysis of the autonomous replication behavior in human cells of the DHFR putative chromosomal origin of replication. *Nucleic Acids Res.* **20:** 5971.

Carroll, S.M., M.L. DeRose, J.L. Kolman, G.H. Nonet, R.E. Kelly, and G.M. Wahl. 1993. Localization of a bidirectional DNA replication origin in the wild type and in episomally amplified murine ADA loci. *Mol. Cell. Biol.* **13:** 2971.

Chittenden, T., A. Frey, and A.J. Levine. 1991. Regulated replication of an episomal SV40 origin plasmid in COS7 cells. *J. Virol.* **65:** 5944.

Delidakis, C. and F.C. Kafatos. 1989. Amplification enhancers and replication origins in the autosomal chorion gene cluster of *Drosophila*. *EMBO J.* **8:** 891.

DePamphilis, M.L. 1989. Transcriptional elements as components of origins of eukaryotic DNA replication. *Cell* **52:** 635.

———. 1993a. Eukaryotic DNA replication: Anatomy of an origin. *Annu. Rev. Biochem.* **62:** 29.

———. 1993b. Origins of DNA replication in metazoan chromosomes. *J. Biol. Chem.* **268:** 1.

———. 1993c. How transcription factors regulate origins of DNA replication in eukaryotic cells. *Trends Cell Biol.* **3:** 161.

DePamphilis, M.L., E. Martínez-Salas, D.Y. Cupo, E.A. Hendrickson, C.E. Fritze, W.R. Folk, and U. Heine. 1988. Initiation of polyomavirus and SV40 DNA replication, and the requirements for DNA replication during mammalian development. *Cancer Cells* **6:** 165.

Dijkwel, P.A. and J.L. Hamlin. 1992. Initiation of DNA replication in the DHFR locus is confined to the early S-period in CHO cells synchronized with the plant amino acid mimosine. *Mol. Cell. Biol.* **12:** 3715.

Ferguson, B.M. and W. L. Fangman 1992. A position effect on the time of replication origin activation in yeast. *Cell* **68:** 333.

Fields-Berry, S.C. and M.L. DePamphilis. 1989. Sequences that promote formation of catenated intertwines during termination of DNA replication. *Nucleic Acids Res.* **17:** 3261.

Forrester, W. C., E. Epner, M. C. Driscoll, T. Enver, M. Brice, T. Papayannopoulou, and M. Groudine. 1990. A deletion of the human β-globin locus activation region causes a major alteration in chromatin structure and replication across the entire β-globin region. *Genes Dev.* **4:** 1637.

Gale, J.M., R.A. Tobey, and J.A. D'Anna. 1992. Localization and DNA sequence of a replication origin in the rhodopsin gene locus of Chinese hamster cells. *J. Mol. Biol.* **224:** 343.

Gilbert, D.M. 1986. The temporal order of replication of *Xenopus laevis* 5S ribosomal RNA genes in somatic cells. *Proc. Natl. Acad. Sci.* **83:** 2924.

———. 1989. "The temporal order of DNA replication in eukaryotic cells: Its relationship to gene expression." Ph.D. thesis, Stanford University, Stanford, California.

Gilbert, D.M. and S.N. Cohen. 1987. BPV plasmids replicate randomly in mouse fibroblasts throughout S-phase of the cell cycle. *Cell* **50:** 59.

Gruss, C. and J.M. Sogo. 1992. Chromatin replication. *BioEssays* **14:** 1.

Gurdon, J.B. 1976. Injected nuclei in frog oocytes: Fate, enlargement and chromatin dispersal. *J. Embryol. Exp. Morphol.* **36:** 523.

Hatton, K.S., V. Dhar, E.H. Brown, M. A. Iqbal, S. Stuart, V.T. Didamo, and C.L. Schildkraut. 1988. Replication program of active and inactive gene families in mammalian cells. *Mol. Cell. Biol.* **8:** 2149.

Handeli, S., A. Klar, M. Meuth, and H. Cedar. 1989. Mapping replication units in animal cells. *Cell* **57:** 909.

Hay, R.T. and M.L. DePamphilis. 1982. 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.

Heck, M.M.S. and A.C. Spradling. 1990. Multiple replication origins are used during *Drosophila* chorion gene amplification. *J. Cell Biol.* **4:** 903.

Heintz, N.H. and J.L. Hamlin. 1982. An amplified chromosomal sequence that includes the gene for dihydrofolate reductase initiates replication within specific restriction fragments. *Proc. Natl. Acad. Sci.* **79:** 4083.

Heintz, N.H. and B.W. Stillman. 1988. Nuclear DNA synthesis in vitro is mediated via stable replication forks assembled in a temporally specific fashion in vivo. *Mol. Cell. Biol.* **8:** 1923.

Hendrickson, E.A., C.E. Fritze, W.R. Folk, and M.L. DePamphilis. 1987. The origin of bidirectional DNA replication in polyoma virus. *EMBO J.* **6:** 2011.

Hyrien, O. and M. Méchali. 1992. Plasmid replication in *Xenopus* eggs and egg extracts: A 2D gel electrophoretic analysis. *Nucleic Acids Res.* **20:** 1463.

Ishimi, Y., R. Ishida, and T. Andoh. 1992. Effect of ICRF-193, a novel DNA topoisomerase II inhibitor, on SV40 DNA and chromosome replication in vitro. *Mol. Cell. Biol.* **12:** 4007.

Karpen, G.H. and A.C. Spradling. 1990. Reduced DNA polytenization of a minichromosome region undergoing position effect variegation in *Drosophila*. *Cell* **63:** 97.

Kornberg, A. and T. Baker. 1992. *DNA replication*. W.H. Freeman, New York.

Krude, T. and R. Knippers. 1991. Transfer of nucleosomes from parental to replicated chromatin. *Mol. Cell. Biol.* **11:** 6257.

Laskey, R.A., S.E. Kearsey, M. Méchali, A.D. Dingwall, A.D. Mills, S.M. Dilworth, and J. Kleinschmidt. 1985. Chromosome replication in early *Xenopus* embryos. *Cold Spring Harbor Symp. Quant. Biol.* **50:** 657.

Leno, G.H., C.S. Downes, and R.A. Laskey. 1992. The nuclear membrane prevents replication of human G2 nuclei but not G1 nuclei in *Xenopus* egg extract. *Cell* **69:** 151.

Liang, C., J.D. Spitzer, H.S. Smith, and S.A. Gerbi. 1993. Replication initiates at a confined region during DNA amplification in *Sciara* DNA puff II/9A. *Genes Dev.* **7:** 1072.

Little, R.D., T.H.K. Platt, and C.L. Schildkraut. 1993. Initiation and termination of DNA replication in human ribosomal RNA genes. *Mol. Cell. Biol.* (in press).

Lusky, M. and M.R. Botchan. 1984. Characterization of the bovine papilloma virus plasmid maintenance sequences. *Cell* **36:** 391.

Mahbubani, H.M., T. Paull, J.K. Elder, and J.J. Blow. 1992. DNA replication initates at multiple sites on plasmid DNA in *Xenopus* egg extracts. *Nucleic Acids Res.* **20:** 1457.

McKnight, S.L. and O.L. Miller. 1977. Electron microscopic analysis of chromatin replication in the cellular blastoderm *Drosophila melanogaster* embryo. *Cell* **12:** 795.

McWhinney, C. and M. Leffak. 1990. Autonomous replication of a DNA fragment containing the chromosomal replication origin of the human c-*myc* gene. *Nucleic Acids Res.* **18:** 1233.

Meier, J., K.-H.S. Campbell, C.C. Ford, R. Stick, and C.J. Hutchison. 1991. Role of lamin LIII in nuclear assembly and DNA replication in cell-free extracts of *Xenopus* eggs. *J. Cell Sci.* **98:** 271.

Nethanel, T.F., F. Slotkin, and G. Kaufmann. 1992. Assembly of SV40 Okazaki pieces from DNA primers is reversibly arrested by ATP depletion. *J. Virol.* **66:** 6034.

Newport, J.W., K.L. Wilson, and W.G. Dunphy. 1990. A lamin-independent pathway for nuclear envelope assembly. *J. Cell Biol.* **111:** 2247.

Orr-Weaver, T.L. 1991. *Drosophila* chorion genes: Cracking the eggshell's secrets. *BioEssays* **13:** 97.

Ravnan, J.-B., D.M. Gilbert, G. Kelly, T. Hagen, and S.N. Cohen. 1992. Random-choice replication of extrachromosomal BPV molecules in heterogeneous, clonally derived BPV-infected cell lines. *J. Virol.* **66:** 6946.

Roberts, J.M. and H. Weintraub. 1988. *cis*-Acting negative control of DNA replication in eukaryotic cells. *Cell* **52:** 397.

Shinomiya, T. and S. Ina. 1991. Analysis of chromosomal replicons in early embryos of *Drosophila melanogaster* by two-dimensional gel electrophoresis. *Nucleic Acids Res.* **19:** 3935.

Simpson, R.T. 1990. Nucleosome positioning can affect the function of a cis-acting DNA element in vivo. *Nature* **343:** 387.

Sugasawa, K., Y. Ishimi, T. Eki, J. Hurwitz, A. Kikuchi, and F. Hanaoka. 1992. Nonconservative segregation of parental nucleosomes during SV40 chromosome replication in vitro. *Proc. Natl. Acad. Sci.* **89:** 1055.

Ustav, E., M. Ustav, P. Szymanski, and A. Stenlund. 1993. Bovine papillomavirus origin of replication requires a binding site for the E2 transcriptional activator. *Proc. Natl. Acad. Sci.* **90:** 898.

Vassilev, L.T. and M.L. DePamphilis. 1992. Guide to identification of origins of DNA replication in eukaryotic cell chromosomes. *Crit. Rev. Biochem. Mol. Biol.* **27:** 445.

Vassilev, L.T. and E.M. Johnson. 1990. An initiation zone of chromosomal DNA replication located upstream of the c-*myc* gene in proliferating HeLa cells. *Mol. Cell. Biol.* **10:** 4899.

Vassilev, L.T., W.C. Burhans, and M.L. DePamphilis. 1990. Mapping an origin of DNA replication at a single copy locus in exponentially proliferating mammalian cells. *Mol. Cell. Biol.* **10:** 4685.

Virta-Pearlman, V.J., P.H. Gunaratne, and A.C. Chinault. 1993. Analysis of a replication initiation sequence from the adenosine deaminase region of the mouse genome. *Mol. Cell. Biol.* (in press).

Vogt, V.M. and R. Braun. 1977. The replication of ribosomal DNA in *Physarum polycephalum*. *Eur. J. Biochem.* **80:** 557.

Wangh, L. 1989. Injection of *Xenopus* eggs before activation, achieved by control of extracellular factors, improves plasmid DNA replication after activation. *J. Cell Sci.* **93:** 1.

Wollman, E.L., F. Jacob, and W. Hayes. 1957. Conjugation and genetic recombination in *E. coli* K-12. *Cold Spring Harbor Symp. Quant. Biol.* **21:** 141.

Wu, C., M. Zannis-Hadjopoulos, and G.B. Price. 1993. In vivo activity for initiation of DNA replication resides in a transcribed region of the human genome. *Biochim. Biophys. Acta* (in press).

Yang, L., J. Mohr, E. Fouts, D.A. Lim, M. Nohaile, and M. Botchan. 1993. The E1 protein of papillomavirus is an ATP dependent DNA helicase and unwinding enzyme. *Proc. Natl. Acad. Sci.* (in press).

Yang, L., J. Mohr, R. Li, F. Nottoli, S. Sun, and M. Botchan. 1991. Transcription factor E2 regulates BPV-1 DNA replication in vitro by direct protein-protein interaction. *Cold Spring Harbor Symp. Quant. Biol.* **56:** 344.

Zhu, J., C.S. Newlon, and J.A. Huberman. 1992. Localization of a DNA replication origin and termination zone on chromosome III of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **12:** 4733.