Origins of DNA replication that function in eukaryotic cells

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This past year has seen a significant increase in our understanding of eukaryotic origins of replication, of the proteins that identify these origins, of DNA sequences that promote their unwinding, and of transcription factors that stimulate origin activity. DNA replication begins at specific sites in both simple and complex genomes, but origins in complex genomes may include nuclear structure as well as DNA sequence.

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Introduction

Analysis 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*), has 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 (reviewed in [1,2]). 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 [simian virus 40 (SV40), polyomavirus (PyV) and plasmids] utilizes the standard replication fork mechanism (Fig. 1): DNA is synthesized continuously on the forward arm but discontinuously on the retrograde arm via the repeated synthesis and joining of short RNA-primed nascent DNA chains (Okazaki fragments). Okazaki fragments themselves may be assembled from a cluster of even shorter RNA–DNA chains of <40 nucleotides [3]. One consequence of the asymmetrical distribution of Okazaki fragments at replication forks is that an origin of bidirectional replication (OBR) is revealed by the transition from discontinuous to continuous DNA synthesis that must occur on each template (Fig. 1), a fact that has been used to identify OBRs in mammalian chromosomes [4,5•,6•]. As the replication fork passes through a nucleosome, the old histone octamer in front of the fork is distributed randomly to either arm [5•,7,8], 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 [9]. As two forks approach one another, topoisomerase II is required for unwinding the final region of duplex DNA separating the forks [10]. Termination of replication does not require specific sequences [11], 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 [12].

In order to understand how DNA replication is regulated one must first determine the nature of origins of replication, what they are and how they work. 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 in simple genomes and encompass from 50 to $\sim$1000 bp of DNA [1,2]. Most, but not all, of these origins have been shown to act as autonomously replicating sequences (ARS) by conferring on other DNA molecules the ability to replicate when transferred to either cells or cell extracts containing the required replication proteins. In the cellular chromosomes of *S. cerevisiae*, replication bubbles have been mapped to within $\pm$ 300 bp of an ARS element and mutations within the ARS core component inactivate the chromosomal origin [11,13•,14•]. Origins of replication have also been mapped to an 0.5–1 kb chromosomal site in *Physarum* [15], and to a 3–5 kb site in *S. pombe* [16], although ARS activity of these origins has not yet been demonstrated. Thus, simple eukaryotic origins appear similar in design to origins of replication in prokaryotic cells, plasmids and bacteriophages [2].

Simple origins

Simple origins have a modular anatomy that includes binding sites for specific recognition proteins and transcription factors. They consist of two basic compo-

Abbreviations

Ad—adenovirus; **ARS**—autonomously replicating sequence; **DUE**—DNA-unwinding element; **EBV**—Epstein–Barr virus; **HSV**—herpes simplex virus; **OBR**—origin of bidirectional replication; **ORE**—origin recognition element; ***ori***—genetic origin of replication; **PV**—papillomavirus; **PyV**—polyomavirus; **SV40**—simian virus 40; **T-ag**—large tumour antigen.

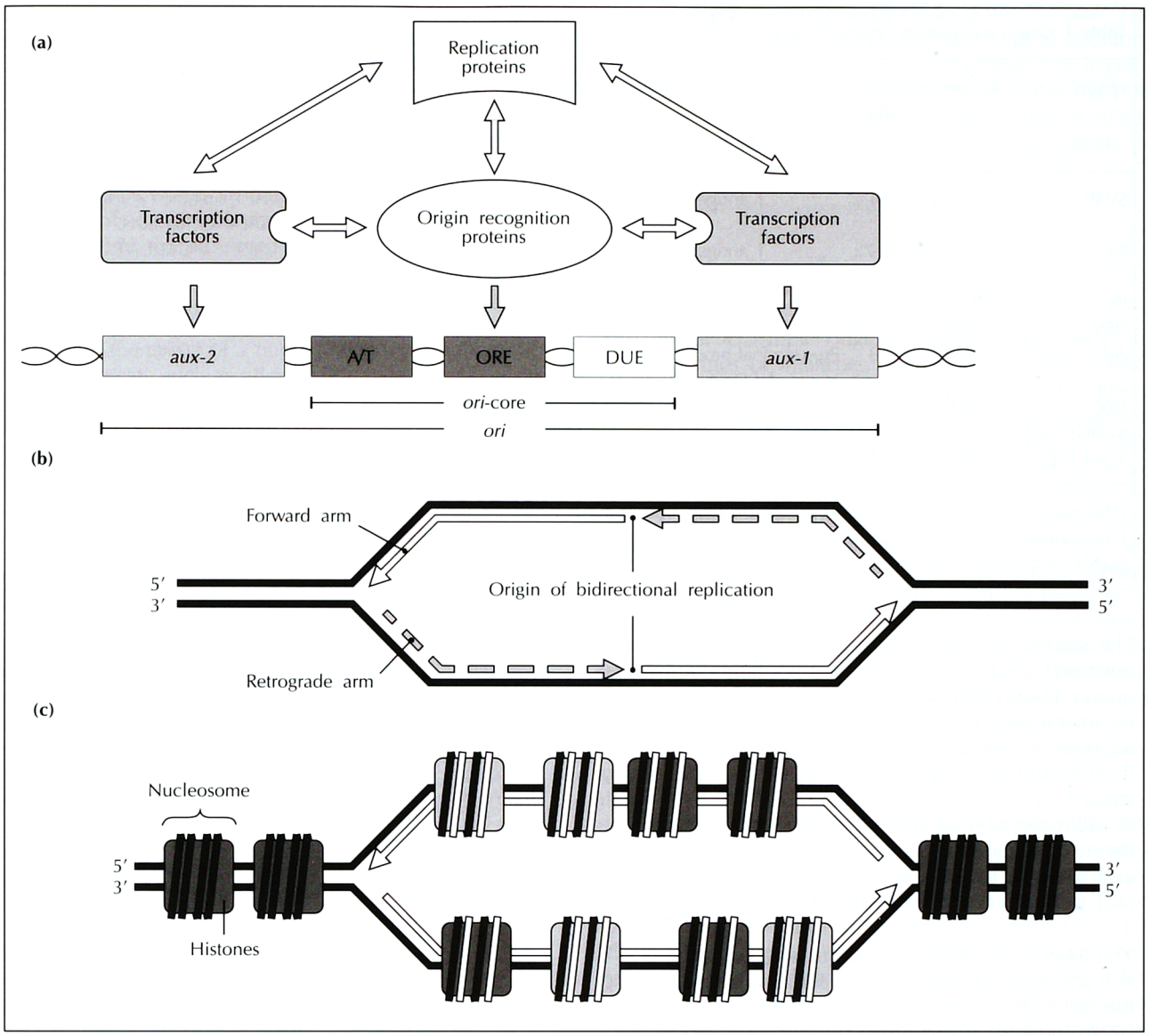

Fig. 1. **(a)** The major components of origins of DNA replication in simple eukaryotic genomes. The genetically defined *ori* consists of an *ori*-core and auxiliary (*aux*) components. The *ori*-core generally comprises an A/T-rich element (A/T), a binding site for OREs, and an easily unwound DNA sequence (DUE). The A/T-rich element is part of the ORE in some origins (Ad and yeast) but distinct in others (SV40, PyV, PV and HSV) and absent in one (EBV). The DUE has been identified in three origins (yeast, SV40 and PyV), and shown to be coincident with the origin of bidirectional DNA replication (OBR) in two of them (SV40 and PyV). The auxiliary components bind specific transcription factors that interact with origin recognition proteins and replication proteins. **(b)** DNA is synthesized continuously on the forward arm (white arrow) but discontinuously on the retrograde arm, via the repeated synthesis and joining of Okazaki fragments (broken grey arrow). Details of chromatin organization at replication forks are found in [9,56•]. **(c)** The arrangement of nucleosomes after DNA replication. Old histones (dark) and new histones (light) are distributed randomly to either arm of the replication fork. Newly replicated DNA is shown as a white arrow.

nents (Fig. 1) [1]: the core component (*ori*-core) and one or more auxiliary components.

Core component

The *ori*-core is the minimal essential *cis*-acting sequence required to initiate DNA replication under all conditions; it is analogous to a transcription promoter. It consists of at least two, perhaps three, elements (Fig. 1). The origin recognition element (ORE) acts as the DNA-binding site for one or more origin-recognition proteins. These proteins (Table 1) are required for initiation of replication. They participate either directly by unwinding the DNA with their helicase activity [17–19] or indirectly through their association with replication proteins such as DNA polymerase and RF-A [20,21] or transcription factors such as PV E2, EBV EBNA-1 and cellular protein NF1 [21–23,24•]. These associations may simply guide replication proteins to *ori*, or they may facilitate the formation or activity of a replication complex.

Table 1. Origin recognition proteins and auxiliary transcription factors.*

Origin*	Reference	Core† (bp)	Origin recognition proteins			Transcription factors‡	
			Protein	Enzymatic activities	Binds to:	Aux-2	Aux-1
SV40	[17,31]	64	T antigen	Helicase, unwinding	DNA pol-α RF-A	AP1, Sp1, NF1 > T-ag (GAL4, VP16, cJUN, GR)	T-ag
PyV	[18,31]	66–72	T antigen	Helicase, unwinding	DNA pol-α	AP1 > > GAL4, VP16 cJUN, E1a, E2, Sp1, (CREB)	T-ag
PV	[19,22,23]	59	E1	Helicase, unwinding	E2	E2	E2
HSV	[59]	67–90	UL9	Helicase		Several candidates	
Ad-2	[21]	18	Pre-terminal protein (pTP)** Ad DNA pol	Prime DNA synthesis DNA synthesis	Ad DNA pol pTP, NF1	NF1, OCT1	
EBV	[24]	114	EBNA-1	(No helicase)	EBNA-1	EBNA-1	
mtDNA (*oriH*)		~90	RNase MRP	Endoribonuclease		mtRNA pol	
Yeast (ARS)	[60,61]	15–35	~6 proteins	?		ABF-1	ABF-1

*The most recent literature citations are in this table and in the text. Additional citations are found in [1,28,29]. †Data are from [1]. ‡Transcription factors in parentheses did not stimulate origin *in vivo*. **Ad origin recognition protein is a complex of pre-terminal protein and Ad DNA polymerase.

The second core element is a DNA region that is easily unwound, the DUE. Although it is not a unique DNA sequence like an ORE, a DUE is determined by base stacking interactions, and is therefore not simply a function of AT content, but depends on nucleotide sequence [25]. In SV40 and PyV origins, the DUE, where large tumour antigen (T-ag) begins unwinding DNA, and the origin of bidirectional replication, defined by the transition between continuous and discontinuous DNA synthesis on each template (Fig. 1), are coincident [1]. Therefore, the DUE probably determines where DNA replication begins.

The third core element is a sequence consisting of a T-rich and an A-rich strand [1]. In some origins (Ad, yeast), this A/T-rich element is part of the ORE. In other origins (SV40 and PyV), it appears to facilitate DUE activity, while in still others (HSV), it may serve as the DUE element itself. This disparity in function, and the fact that in the EBV origin it is not even present, makes it difficult to assign a specific role for the A/T-rich element.

Ori function is usually critically dependent on the spacing, orientation and arrangement of these three core elements [1,26]. In general, there is a lack of symmetry in both the organization of sequence elements and their specific functions in replication origins, and as a result, DNA synthesis initiation events within *ori*-core occur only on one DNA strand (e.g. Ad, mitochondrial DNA, SV40, PyV and PV). Therefore, initiation of bidirectional replication at origins such as SV40, PyV and PV is unlikely to be a symmetrical event with two forks moving simultaneously out of the ORE. A more likely scenario is that forward arm synthesis begins on one strand of *ori*-core, and then progresses beyond *ori*-core before DNA synthesis is initiated in the opposite direction on the complementary strand (see the initiation zone model in [27]).

Auxiliary components

Auxiliary components affect the efficiency but not the mechanism of replication. They consist of binding sites for transcription factors that increase *ori*-core activity from 2- to 1000-fold, depending on the origin and on experimental conditions (reviewed in [1,28,29••]). As they are dispensable under some conditions, they are analogous to, and sometimes identical to, transcription enhancers. In some cases (SV40, PyV and EBV), the same sequence elements function both as auxiliary components in replication and promoters or enhancers in transcription; *cis*-acting mutations that affect one process also affect the other. Transcription enhancers generally act independently of their distance and orientation relative to the promoter. In contrast, orientation and spacing between auxiliary sequences and *ori*-core are critical in some origins (Ad, SV40 and PyV [21,30,31]), while in other origins (EBV and yeast [24•,32•]) these parameters are flexible. These differences presumably reflect differences in the specificity and strength of interactions between transcription factors and origin recognition proteins, as well as differences in the mechanism by which *ori*-auxiliary components function.

The activity of *ori*-auxiliary components requires that they bind one or more transcription factors with specific activation domains (Table 1). However, the activation domain for DNA replication is not necessarily the same activation domain that stimulates DNA transcription, and there is no correlation between the ability of a transcription factor to stimulate promoter activity and its ability to stimulate origin activity. For example, the transcription activation domains of NF1, OCT1 and E2 are not required to activate Ad or PV *ori*-core [21,33,34], and while the transcription activation domains of VP16 and cJUN stimulate transcription strongly, they stimulate

PyV *ori*-core weakly and have no effect on SV40 *ori*-core [31,35]. The specific requirements for activation domains and spatial arrangements of *ori* components suggest that proteins bound to auxiliary components interact specifically with proteins bound to ORE. Such interactions have been demonstrated with Ad, PV and EBV origins (Table 1) [21–23,24•]. The ability to stimulate a particular origin may be limited to specific members of a transcription factor family. For example, the transcription activation domain of cJUN, the prototype of the AP1 family, does not stimulate SV40 *ori*-core even though AP-1 DNA-binding sites can completely substitute for the normal SV40 *aux-2* region composed of Sp1 and T-ag binding sites [31]. Moreover, the ability of a unique transcription factor activation domain such as cJUN to stimulate the same *ori*-core can vary with cell type, suggesting the involvement of co-activator proteins in replication as well as transcription. Thus, as the abundance of specific transcription factors varies among cell types, the same transcriptional elements can have a much stronger effect on one process relative to the other, depending on cell type.

Transcription factors can stimulate an *ori*-core by at least four different mechanisms [1,2,29••]. In the first mechanism, transcription through *ori*-core is followed by site-specific cleavage by an endoribonuclease, which can generate RNA primers for initiation of DNA synthesis by the appropriate DNA polymerase. Examples are *oriH* mitochondrial DNA, *E. coli* filamentous and T-odd phages and *E. coli* plasmid ColE1. In the second mechanism, transcription factors also can facilitate binding of origin recognition proteins to *ori*-core. For example, NF1 (a member of the CTF family [36]) facilitates binding of subsaturating concentrations of the Ad2 pre-terminal protein–Ad DNA polymerase complex [21], and the PV-encoded enhancer specific activation protein, E2, facilitates binding of E1 to the PV ORE [22,23]. The third mechanism utilizes transcription factors to facilitate the activity of an initiation complex after it has formed. For example, SV40 *ori*-auxiliary components stimulate SV40 *ori*-core by facilitating T-ag dependent DNA unwinding at *ori*-core [37]. OCT1 may perform the same function at the Ad2 *ori*, as OCT1 binding to the Ad2 *ori* does not affect binding of the Ad2 pre-terminal protein–Ad DNA polymerase complex, but does change DNA conformation [33]. In the fourth mechanism, transcription factors can prevent chromatin structure from interfering with binding of initiation factors to *ori*-core, much as they have been shown to do with promoters. The best candidate for this mechanism is PyV, where the PyV enhancer (*aux-2*) is dispensable under conditions in which a repressive chromatin structure appears to be absent [38,39•]. This mechanism has also been suggested for SV40 *aux-2* on the basis of the ability of NF1 or GAL4:VP16 [40] to prevent chromatin assembly from interfering with SV40 DNA replication *in vitro*. However, while NF1 DNA-binding sites also stimulated SV40 *ori*-core activity *in vivo*, GAL4:VP16 DNA-binding sites, in the presence of GAL4:VP16 protein, did not [31,35]. Therefore, the significance of GAL4:VP16 action on DNA replication *in vitro* is questionable. Furthermore, simply pre-binding T-ag alone to SV40 *ori* prevents nucleosomes from repressing replication (C Gruss, J Wu, T Koller, JM Sogo, unpublished data) [41]. Thus, the critical question of whether proteins bound to *aux-2* facilitate T-ag in preventing repression during nucleosome assembly has not yet been answered. Transcription factors such as ABF1 can stimulate yeast origins [32•], but their mechanism is not yet known.

Complex origins

In contrast to simple origins, origins of replication in chromosomes of metazoan animals appear more complex (reviewed in [1,42••]). The most dramatic difference is that ARS elements that function in metazoan cells are illusive, at best [43]. In fact, virtually any DNA can replicate in frog eggs, and initiation occurs, on average, at one randomly chosen site in each DNA molecule [44,45]. Replication bubbles appear to be randomly distributed throughout the tandem repeats of histone genes in early *Drosophila* embryos [46]. Such results suggest that initiation of replication in metazoa is not tightly coupled to specific DNA sequences. However, promiscuous selection of initiation sites may be unique to rapidly cleaving embryos and to plasmid DNA replication. The critical question is whether or not differentiated metazoan cells initiate DNA replication at specific sites in native chromosomes, and, if so, under what conditions do the sequences in these sites activate replication.

Several methods have been developed for mapping sites where replication begins in metazoan chromosomes [47]. Analyses of nascent DNA strands, recognized by incorporation of labeled nucleotide precursors, have mapped initiation sites in the hamster dihydrofolate reductase gene region (*ori*-β), the mouse *c-myc* gene region, the mouse adenosine deaminase gene region, the *ORS*-3 locus in human cells, and the hamster rhodopsin gene locus [1,4,5•,6•,42••,48]. These studies included identification of the earliest labeled DNA fragments, trapping replication bubbles at the site of their origin, measuring the lengths of unique nascent DNA strands, and identification of the transitions from continuous to discontinuous DNA synthesis that define an OBR (Fig. 1). The data reveal that DNA replication in mammalian chromosomes occurs by the same replication fork mechanism used in simple genomes, and that most (at least 80%) initiation events originate bidirectionally from a specific site as small as 0.5 kb but no larger than 5 kb in size. Potential origin recognition proteins have also been identified [47].

Analyses of total DNA for the presence of replication bubble and fork structures by their mobilities during two-dimensional gel electrophoresis reveals an apparent paradox. In the same hamster dihydrofolate reductase *ori*-β region where nascent DNA strand analysis revealed a specific OBR, replication bubbles were observed to be distributed essentially randomly throughout the entire intergenic region (~55 kb) with equivalent numbers of replication forks traveling in both directions [49]. Simi-

larly, replication bubbles were distributed throughout the 31 kb non-transcribed intergenic region in human rDNA genes (RD Little, THK Platt, CL Schildkraut, unpublished data).

The simplest explanation to this paradox is that DNA contains many possible sites where replication can initiate, but the effects of chromosome structure and nuclear organization suppress initiation at most of these sites while facilitating initiation at the OBR (Fig. 2). This model is reminiscent of the Jesuits' dictum that 'many are called, but few are chosen', and is based on the fact that published two-dimensional gel data detects replication structures without determining the fraction of initiation events that occurs in any one DNA segment, while nascent DNA strand analyses provide a quantitative assessment. 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 [50•,51]. Chromatin structure can suppress access to alternative origins, while nuclear structure may be required to promote DNA unwinding [52], generating specific sites (OBRs) where DNA is threaded through a helicase complex, such as occurs at the SV40 origin [17]. 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 [53•]. 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.

Support for the Jesuit Model also comes from two-dimensional gel analyses of replication events at DNA loci that undergo programmed amplification during animal development. Amplification of the *Drosophila* chorion gene locus is regulated by five *cis*-acting sequence elements located within ~8 kb of DNA. Replication bubbles were found distributed throughout the amplified region, but ~80% of the initiation events occurred within a 1 kb region of DNA encompassing one of the *cis*-acting elements [54,55]. Similarly, in *Sciara* replication bubbles at an amplified region were found distributed throughout a 6 kb zone, but >90% were initiated within a ~1 kb area of that zone [56•]. Thus, during programmed amplification, most initiation events occur at a specific site (possibly a *cis*-acting element) equivalent in size to the OBRs mapped in mammalian cells by nascent DNA strand analyses, although some initiation events can occur at other sites throughout the initiation zone.

Conclusion

The evidence is now compelling that initiation of DNA replication begins at specific sites in the chromosomes

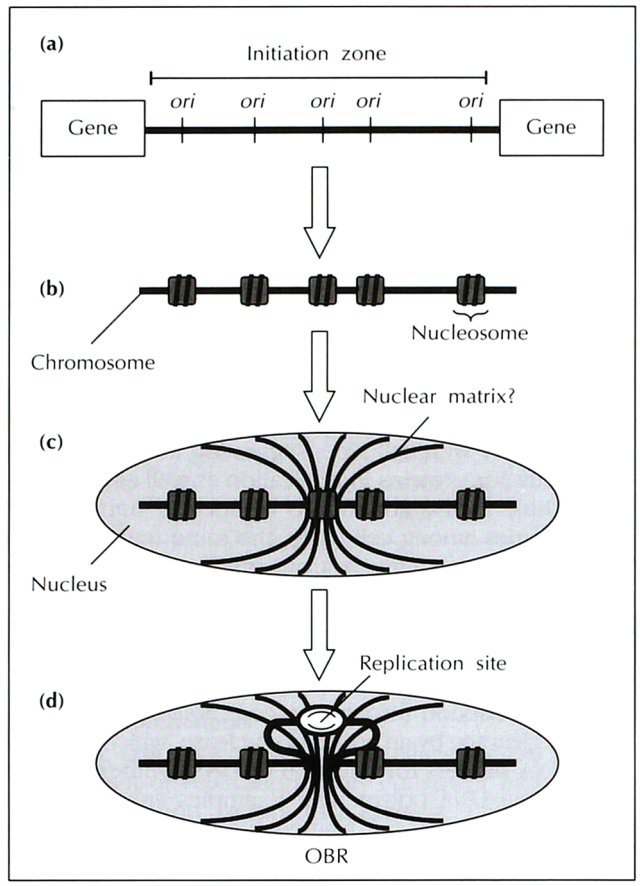

Fig. 2. Jesuit model of origins of replication in metazoan chromosomes. **(a)** DNA contains many sites where replication can begin (*ori*). **(b)** Schematic representation of the chromosome containing *ori* sites; note that the positioning of one *ori* site per nucleosome does not necessarily reflect the actual arrangment. **(c)** Chromosomal structure and organization of unidentified components of the nuclear matrix may repress some *ori* sites, while activating others (the central site in this figure). **(d)** This initiates replication at the origin of bidirectional replication (OBR).

of both simple and complex organisms, that bidirectional replication from these origins uses the standard replication fork mechanism, and that nucleosome segregation is distributive. Moreover, the anatomy of eukaryotic origins has come into sharper focus. Simple origins consist of well defined sequence elements that occupy from 18 bp (Ad4) to ~1000 bp (EBV). These elements include a binding site (ORE) for one or more proteins that specifically recognize the origin, a DNA structure that promotes unwinding (DUE), and transcription factor binding sites that facilitate origin activity. Complex origins in metazoan chromosomes are less well defined. Genetically required origin components have been demonstrated convincingly only during programmed gene amplification. However, both during gene amplification and S phase, most initiation events originate from a site comparable in size to simple origins (0.5–3 kb, the OBR), but some initiation events occur throughout a much broader initiation zone (6–55 kb) surrounding the OBR. The initiation zone may consist of entire intergenic regions, but no initiation events have been detected within genes. Metazoan ori-

gins may include both specific DNA sequences as well as chromatin structure and nuclear organization, something that may be difficult to produce with plasmid DNA. Future progress will depend on finding a functional assay for origin activity that exhibits the same characteristics observed *in vivo*.

Note added in proof

Xenopus egg extracts have recently been observed to initiate DNA replication at one of the same initiation sites (DHFR ori-β) used by hamster cells *in vivo*, provided that the DNA substrate is presented to the extract as isolated nucei rather than bare DNA (D Gilbert, H Miyazawa, M DePamphilis, unpublished data). This result provides direct support for the concept that site-specific initiation of replication in metazoan cell chromosomes is determined by nuclear organization.

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