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Review

New beginnings in studies of eukaryotic DNA replication origins

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I. Introduction

DNA fiber autoradiographic studies carried out over 20 years ago first suggested that eukaryotic DNA replication begins at discrete sites spaced at irregular intervals along chromosomal DNA molecules [1]. The discrete initiation sites were called 'origins'. Later on, the DNA replicated from a single origin was termed a 'replicon' (reviewed in Ref. 2). At the time, it was impossible to determine whether these origins and replicons corresponded to specific nucleotide sequences or were randomly located (with different origins being used in different cells). In the absence of accurate

information, considerable speculation developed about possible relationships between origin (or replicon) location and embryonic development, regulation of gene expression, time of replication, and boundaries of topological domains within chromosomes. Recently developed origin and replicon mapping techniques now allow distinction between random and specific origins, and results employing these techniques are beginning to emerge.

The results which are already available allow some preliminary conclusions to be drawn. Studies in numerous eukaryotic organisms suggest the existence of preferred initiation sites. The most advanced studies, carried out in the yeast, *Saccharomyces cerevisiae*, have progressed to the point where the nucleotide sequences required for origin function can be partially specified, stimulating a quest for proteins which recognize these sequences. New experimental approaches have revealed that a DNA sequence element with a low free energy for unwinding is an essential and evolutionarily conserved component of yeast and prokaryotic replication

Abbreviations: *ARS*, autonomously replicating sequences; *DUE*, DNA unwinding element; *DHFR*, dihydrofolate reductase; *IVR*, in vitro run off.

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origins [3-5], permitting proposal of a model for the role of such sequence elements in initiation. We intend, in this review, to summarize the current results and to present our own preliminary conclusions as to their significance.

This review focuses on chromosomal and chromosomally-derived replication origins. No attempt has been made to incorporate the wealth of available information on eukaryotic viral replication origins.

II. Localization of yeast replication origins

ARS elements in yeasts

The advantages which make yeasts favorite organisms for study of many areas of cell biology and biochemistry also make them useful for studying DNA replication [6]. Their small genome size and short generation time render them particularly useful for replicon mapping techniques based on detection of signals from replicating molecules by molecular hybridization (see below). The property of yeasts which makes them most useful for study of replication origins is the fact that, in yeasts, plasmids containing origins can replicate without integration into chromosomal DNA. In 1979 it was found that certain sequence elements derived from yeast (*S. cerevisiae*) chromosomal DNA would allow plasmids containing these elements to transform recipient yeast cells at remarkably high frequency, and the plasmid sequences in the successfully transformed cells were not integrated into chromosomal DNA [7]. The short sequence elements were called *autonomously replicating sequences* or *ARS elements* [7]. Because genetic tests have revealed that *ARS elements* promote efficient plasmid replication [8,9], it has been widely assumed that *ARS elements* are replication origins. However, until recently there was no direct evidence for this assumption; *ARS elements* might have increased replication efficiency in some other way, for example by acting as binding sites for topoisomerases. Evidence that *ARS elements* can, indeed, function as replication origins has now been provided by new physical techniques for replicon mapping [10-12].

Partial correspondence between origins and ARS elements

The new physical techniques referred to above are two-dimensional (2D) gel electrophoretic analyses of replication-fork-containing restriction fragments. One technique exploits the abnormal migration of non-linear restriction fragments under certain electrophoresis conditions [10]. The second technique employs a denaturing second dimension to permit analysis of the DNA sequence content of nascent strands of different sizes [11,12]. Use of both techniques together permits unambiguous determination of directions of replication fork

movement and locations of origins and termination sites in any stretch of DNA for which suitable hybridization probes are available [13,14].

The 2D gel techniques were initially applied to localization of origins and termini in the yeast 2 μ m plasmid [10,11] and in an *ARS1*-containing plasmid [10]. In both cases, the plasmids appeared to be replicated from a single origin located, within experimental error of several hundred basepairs, at the single *ARS* element in each plasmid. These findings showed that the prevailing hypothesis, that *ARS elements* are origins, is probably correct for the two plasmids investigated. The next question was, do *ARS elements* also serve as origins during chromosomal replication? The results obtained so far suggest that the answer to this question is not a simple one.

The first indication that *ARS elements* in chromosomes might not always serve as replication origins came from studies on the ribosomal DNA (rDNA) of yeast. This DNA consists of about 120 identical tandemly repeated units, each 9.1 kb in length (Fig. 1A [15]). Each repeat contains a transcription unit for the 5 S rRNA and a separate transcription unit for the 37 S precursor RNA which is processed into the 5.8 S, 18 S, and 25 S rRNAs. The two transcripts are separated by two nontranscribed spacers (NTS1 and NTS2). A weak *ARS* element is present in NTS2 [16]. Results obtained by Linskens and Huberman with both 2D replicon mapping techniques [13] suggest that replication usually begins at a position within the previously-mapped *ARS* element and proceeds bidirectionally (Fig. 1B, 1, 2). Leftwards-moving forks stop when they reach position 7.0 kb (just to the right of the *HindIII* site near the termination site of 37 S transcription; Fig. 1B, 2-4). Rightwards-moving forks continue replication in the direction of 37 S RNA transcription until they meet stalled leftwards-oriented forks (Fig. 1B, 4, 5). Rightwards-moving forks usually proceed through 3-10 repeat units before encountering stalled leftwards-oriented forks. Therefore, rDNA replication is predominantly unidirectional and *most of the ARS elements are not used as origins* in any single S phase. Stalling of leftwards-moving replication forks near position 7.0 and infrequent usage of *ARS elements* as origins have also been observed by B. Brewer and W. Fangman (unpublished data).

Additional evidence that *ARS elements* do not always serve as origins in chromosomes has been produced by studies of yeast chromosome III. Fig. 2 presents a diagram summarizing current replicon mapping studies at the left end of this chromosome. Initial results demonstrated that the strong *ARS* element in the A6C *BamHI* fragment (the A6C *ARS* [17,18]) appeared to serve as a replication origin [14]. In the cell line used in these first studies, the A6C fragment appeared to be replicated entirely from its internal origin [14]. Subse-

Fig. 1. Model for replication of the rDNA of *S. cerevisiae* (A) Diagram of a single, 9.1 kb long, repeat unit. Nucleotide numbering starts at the beginning of the 37 S precursor. Transcription of this precursor (from 1 to 6570) is indicated by the thin, wavy vertical lines which represent nascent RNA molecules. The 5 S gene is located between 7715 and 7835. There are two nontranscribed spacer regions, NTS1 (6569 to 7714) and NTS2 (7836 to 9084). The ARS element is a *BsuI*-*AluI* fragment located between 8357 and 8928. Restriction sites are indicated for *HindIII* (H at 458 and 6855), *BglI* (B at 1288), and *MluI* (M at 3877). (B) Schematic diagram of the replication of several repeat units. ARS elements and 37 S transcription are indicated as in section (A). This figure is modified from [13]. See text for further details.

quent studies with different cell lines and with different growth temperatures (Linskens, M.H.K. and Huberman, J.A., unpublished data) show that, in some cases, the A6C fragment can also be replicated by forks from external origins. However, the internal origin always accounts for the majority of replication. Two additional ARS elements on chromosome III (both near the centromere), the C2G1 ARS and the J11D1 ARS [17,18] also function as origins a fraction of the time (Greenfeder, S. and Newlon, C.S., personal communication).

More recent studies, (Davis, L.R. and Huberman, J.A., unpublished data) in which replicon mapping has been pursued towards the telomere from the A6C origin (Fig. 2), suggest that *several ARS elements do not function as origins in their chromosomal context*. The leftwards-moving forks which start at the A6C origin appear to continue at least to within 2 kb of the left end of

the chromosome (Fig. 2). The forks may go farther, all the way to the end, but the terminal *BamHI* fragment has not yet been analyzed. The results obtained so far do not exclude the possibility that the leftwards-moving forks might stall near the junction between the J10A and *HML*-containing *BamHI* fragments, with replication continuing in the same direction due to initiation at the right-most ARS in the *HML*-containing fragment (suggested as an alternative in Fig. 2). Further experiments will distinguish between these possibilities. In either case, it is already clear that neither of the two strong ARS elements associated with mating type cassette "silencer" sequences to the left and right of *HML* [19] nor the weak ARS element in the D10B *BamHI* fragment [17] serves as an origin in the chromosome. Another ARS element, apparently inactive as an origin, may be present at the telomere (indicated with a '?' in Fig. 2). The left telomere of chromosome III lacks a

Fig. 2. Several *ARS* elements near the left end of yeast chromosome III do not function as origins. The diagram shows the *Bam*HI fragments near the left end of chromosome III cloned by Carol Newlon and her collaborators ([17] and unpublished results). *Bam*HI sites are indicated by B. The names of the rightmost 5 fragments [17] are indicated above the fragments. Strong *ARS* elements are indicated by black boxes, and the weak *ARS* element in D10B is indicated by a shaded box. See text for further details.

type Y' *ARS*-like region but possesses a type X *ARS*-like region [20]. This *ARS*-like region contains a perfect match to the *ARS* consensus sequence but has not been directly tested for *ARS* function [20]. Regardless of whether the type X telomeric *ARS*-like region functions as an *ARS* element in plasmids, available data suggest that it is unlikely to be active as an origin in the chromosome: so far, no evidence for replication forks entering the telomere-adjacent *Bam*HI fragment (Fig. 2) at its telomere-proximal end has been detected.

In comparison to the (admittedly preliminary) investigations of *S. cerevisiae* described above, studies of the fission yeast, *Schizosaccharomyces pombe*, lag much further behind. *ARS* elements can be detected in *S. pombe* [21,22], as they can in *S. cerevisiae*. A recent study (Zhu, J. and Huberman, J.A., unpublished data) shows that at least one of these *S. pombe* *ARS* elements (No. 772; [21]) is located close to or at a chromosomal origin. However, this chromosomal origin functions only about 1/3 of the time; most of the time the *Eco*RV fragment containing the No. 772 *ARS* element is replicated from (an) external origin(s).

Thus, although the number of chromosomal regions investigated is still very small, the available results suggest that the relationship between *ARS* elements and chromosomal origins will prove to be a complex one. The evidence suggests that specific sequences are, indeed, preferred for use as chromosomal origins (i.e., origins are not chosen completely randomly), but that many of these preferred origins are not active in every S phase; the probability that any particular preferred origin sequence will 'fire' in a particular S phase is different for different origin sequences. It seems possible that most or all of these preferred origins will prove to have *ARS* activity when assayed in plasmids, but at

the same time it seems likely that a large portion of the chromosomally-derived sequences which have *ARS* activity in plasmids will prove to be inactive or only partially active as origins in their chromosomal context.

What types of effect might "chromosomal context" have on *ARS* elements in chromosomes? Results to date suggest that in some cases chromosomal context has no significant effect (in these cases *ARS* elements function as chromosomal origins), and in other cases it has a negative effect (in these cases the functioning of *ARS* elements is reduced or prevented). Positive effects of chromosomal context have not yet been detected. However, we see no reason why they should not exist. Strong positive chromosomal context effects might lead to origin function by sequences which do not have *ARS* function (that is, some chromosomal origins may prove to be located at sites which do not have *ARS* activity when assayed in plasmids).

How might "chromosomal context" exert a negative effect on an *ARS* element? If replication were to initiate at a neighboring origin, a fork from that origin might replicate the *ARS* element before the *ARS* element could itself function as an origin. The controls that prevent more than one round of replication during a single S phase (see section VII) would then prevent the *ARS* element from functioning as an origin during the completion of that S phase. Transcription may also produce a negative effect. Transcription through a plasmid-borne *ARS* inhibits its function [23]; therefore, it is possible that transcription through an *ARS* element on a chromosome might prevent that *ARS* from working as an origin. In addition, the probability of origin function might depend on whether the *ARS*-containing region is in a condensed or open chromatin configuration or on where the *ARS*-containing region is located

in the nucleus. Finally, there may be *cis*-acting sequences which suppress the origin activity of nearby *ARS* elements. Either these hypothetical sequences would have to be capable of acting over a distance of several kb or more (because they must have been separated from each known *ARS* element during construction of the chimeric plasmid which allowed its identification as an *ARS* element) or these *cis*-suppressors are inactive on circular plasmids. All of these possible explanations can be tested by appropriate biochemical or genetic experiments.

III. DNA sequence requirements of yeast *ARS* elements

The DNA sequence requirements for *ARS* function have been extensively studied. An early comparison of *ARS* elements revealed an 11 bp consensus sequence common among the *ARS* elements: (A/T)TT-TATRTTT(A/T) [24]. Construction and functional analysis of point mutations in this *ARS* 'core consensus' sequence subsequently confirmed the significance of the sequence for DNA replication [25]. The core consensus sequence is the only component of *ARS* elements in which limited base substitutions significantly reduce *ARS* function [25,26–28]. The data are consistent with the frequently proposed role of the core consensus sequence as a protein recognition element. The protein which recognizes this required sequence is anticipated to be an 'initiator' protein, by analogy to well-studied prokaryotic replication origins (reviewed in Ref. 6).

The core consensus sequence is unable to promote autonomous replication of plasmids on its own. Efficient initiation of DNA replication requires in addition a flanking sequence located 3' to the T-rich strand of the core consensus [26–29]. This flanking region has been called a 'replication enhancer' [30–32]. The term 'enhancer' may be an unfortunate choice, since the element does not appear to be position-independent like a transcriptional enhancer. Specifically, sequences which stimulate *ARS* activity have been found only 3' to the T-rich strand of the consensus, while 5' sequences have negligible or negative effects [28,29]. In addition, insertion mutants, in which the 3' flanking region is moved significantly downstream, are replication deficient [18,33].

In contrast to studies of the core consensus, sequence comparisons and mutational analyses have been less revealing about the role of the flanking sequence. Although high in A + T content, the flanking sequence exhibits little primary sequence homology from one *ARS* to another [24]. Furthermore, the region tolerates a variety of substitutions, insertions and deletions in the required region [25–28]. In fact, a comprehensive linker-scanning analysis of the *ARS* near the copy 1 histone H4 gene (H4 *ARS*) detected no specific primary

sequence requirements within the essential flanking region [27]. Bouton and Smith [27] proposed that the H4 *ARS* flanking sequence contains multiple copies of a sequence element which stimulates *ARS* function. The authors reasoned that replication-deficient deletion mutants had fewer than the minimal number of copies of this stimulatory sequence remaining, while replication-competent 10 bp linker-scanning mutants retained more than the minimal number of copies. The repeated element must not have a strict sequence motif since a repeated sequence is not obvious by inspection. A somewhat more specific version of this hypothesis was put forth by Palzkill and Newlon [28]. These authors have suggested that the flanking sequence does in fact contain a repeated sequence which consists of near matches (10/11 or 9/11 bp) to the core consensus sequence. They propose that the near matches also bind the initiator protein, by analogy to the multiple initiator binding sites present in the *E. coli* replication origin (*oriC*). However, we note that, unlike the multiple initiator binding sites of enterobacterial replication origins [34], the number and spacing of the near matches is not consistent among *ARS* elements. Furthermore, only the exact match at the core consensus sequence exhibits the sensitivity to point mutations demonstrated by the multiple initiator binding sites of prokaryotic origins [35]. The crucial test of the Palzkill and Newlon hypothesis (binding to the near matches) awaits the isolation of the putative initiator protein.

The above proposals suggest that protein recognition plays the primary role in mediating the function of the *ARS* 3' flanking region. An additional suggested role for the 3' flanking sequence is DNA bending. It has been demonstrated that the flanking region of *ARS1* includes a sequence which assumes a bent DNA conformation [36]. A potential significance of DNA bending at yeast replication origins has been proposed [30,36] by analogy to its significance in the bacteriophage λ replication origin [37]. However, DNA bending in the λ origin appears to facilitate the interaction of multiple functional copies of the λ consensus sequence with the initiator protein. In contrast, *ARS* elements contain only one core consensus sequence as defined by sensitivity to point mutations. Consequently, a requirement for DNA bending in the *ARS* is not obvious. Furthermore, there is no bent DNA sequence motif in the 2 μ m plasmid *ARS*, and the bent sequence in the H4 *ARS* can be deleted without a detectable decrease in high frequency transformation [27,30]. The mutations which were used to implicate the importance of the bent DNA at *ARS1* were large deletions which might alter additional properties of the origin (see below). The effects of the smaller deletions on replication efficiency could only be detected when the carbon source was galactose [36]. A more recent attempt to substitute oligonucleotides with bent DNA properties for the *ARS*

flanking sequence showed some success [30]. However, the primary sequences which impart DNA bending are not clearly distinct from sequences that confer a low energy for DNA unwinding (see below).

A novel explanation for the sequence requirement of the *ARS* 3' flanking region has been proposed by Umek and Kowalski [3]. This explanation rests upon the characterization of the free energy requirement for unwinding *ARS* elements. While examining the unwinding properties of naturally-occurring DNA sequences, they discovered that yeast replication origins are readily unwound in supercoiled plasmids [3,38]. Unwinding was detected through the formation of a single-strand-specific-nuclease-hypersensitive site. The authors proposed that the unwound, nuclease-hypersensitive sequence is the critical site for origin unwinding during initiation. Consistent with this proposal, the nuclease-hypersensitivity assay correctly predicted the DNA sequence unwound by the initiator protein at the *E. coli* chromosomal origin of replication (*oriC*) ([4,39]; Eddy, M.J. and Kowalski, D., unpublished results). The readily unwound *oriC* sequence is situated asymmetrically with respect to the sequence containing the initiator binding sites. Similarly, *ARS* unwinding maps to the essential A + T-rich region flanking the *ARS* core consensus sequence (the presumed initiator binding site). Easily unwound sequences, such as the ones in *oriC* and in *ARS* elements, are high in A + T content, but ease of unwinding cannot be predicted from A + T content alone [38]. Thus, as in DNA melting [40], certain easily unwound regions may occur in sequences of average A + T content. The easily unwound regions of *ARS* elements include runs of A and T as found in bent DNA sequences [41]. Deletion mutations which remove runs of A and T may alter the unwinding properties of the region as well.

Umek and Kowalski have demonstrated that formation of the nuclease-hypersensitive site correlates with replication efficiency in a series of linker-scanning, linker-deletion and external deletion *ARS* mutants [3]. Nuclease hypersensitivity is interpreted to reflect a low free energy requirement for DNA unwinding. Consistent with this interpretation, a biologically unrelated nuclease-hypersensitive sequence from pBR322 restored unwindability and origin function to replication-deficient *ARS* deletion mutants [3]. The results suggest that the *ARS* flanking sequence influences the initiation of DNA replication by determining the energy required for origin unwinding: the most efficient *ARS* elements would have flanking sequences requiring the least energy to unwind.

More recently, Umek and Kowalski have directly demonstrated the low free energy requirement for *ARS* unwinding through use of 2D electrophoresis of plasmid topoisomers (unpublished data). Stable origin unwinding in vitro proved to be temperature-dependent: un-

winding was detected at 37°C but not 23°C. Thus, thermal energy contributes to the energy requirement for origin unwinding in vitro. Consistent with a thermal energy contribution to origin unwinding in vivo, mutant *ARS* elements with increased energy requirements for unwinding replicate efficiently at 30°C but less efficiently at 23°C (Umek and Kowalski, unpublished data). That is, certain large deletions of *ARS* flanking sequence generate cold-sensitive *ARS* mutations. The data are consistent with the detection of temperature-dependent DNA unwinding at the *ARS* and the proposal of Umek and Kowalski that DNA unwinding is the primary role of the *ARS* flanking sequence. The results are opposite to those predicted if the flanking sequence functioned through DNA bending, since reduced temperatures stabilize DNA bending [41] and therefore would be expected to enhance replication efficiency. Furthermore, temperature-dependent initiation of DNA replication is not a predictable consequence of the protein binding models discussed above, since slight reductions in temperature would not be expected to significantly disrupt protein-DNA interactions. Thus, unwinding the *ARS* flanking sequences of *S. cerevisiae* appears to be a regulatory step in the initiation of replication. Thermal energy (determined by cell growth temperature) contributes to origin unwinding and *ARS* function. Presumably other potential unwinding mechanisms (DNA unwinding proteins, DNA binding proteins and/or torsional stress) also contribute to origin unwinding in vivo.

ARS elements from *S. pombe* have recently been isolated and sequenced [21,22]. All nine *ARS* elements isolated so far contain at least a 10/11 match to an 11 bp consensus: (A/T)RTTTATT(A/T). However, specific deletion of the *S. pombe* consensus sequence resulted, where tested, in only minimal reductions in *ARS* efficiency [21,22]. Thus, either the *S. pombe* consensus sequence identified by computer search is not a core consensus analogous to that of *S. cerevisiae* or the initiation event in *S. pombe* differs from that in *S. cerevisiae* in not requiring such a core consensus. Like *S. cerevisiae* *ARS* elements, *S. pombe* *ARS* elements are generally A + T-rich. Thus, *S. pombe* *ARS* elements may also contain a DNA sequence which stimulates initiation via a low energy requirement for unwinding, as found in *S. cerevisiae* *ARS* elements.

IV. The search for the initiator protein in *S. cerevisiae*

The descriptions of the DNA sequence organization of yeast replication origins discussed above include a role for the core consensus sequence. The conservation of this consensus among *ARS* elements has led to the expectation that the core consensus sequence binds an initiator protein, probably as the first step in the initiation process. However, despite considerable experi-

mental effort, such an initiator protein has not yet been isolated. The efforts to isolate the initiator protein include both genetic and biochemical approaches.

Genetic approaches to isolating the gene encoding the initiator protein have concentrated on identifying *trans*-acting mutations which alter the replication efficiencies of wild-type or mutant *ARS*-containing plasmids. One approach has been to identify mutants which reduce the mitotic stability of *ARS*-containing plasmids [42]. Although mutants which possess the desired phenotype have been identified, further characterization is required to determine whether these mutants encode gene products directly involved in the initiation of DNA replication. An alternative approach has been to identify mutant yeast strains which improve the replication efficiency of *ARS* mutant derivatives which are replication inefficient in wild-type cells [43]. Kearsey and Edwards have used this approach to implicate a number of separate loci in the initiation event. One of the identified genes is essential, and a temperature-sensitive mutation has facilitated isolation and sequence analysis of this gene (*RAR1*). The DNA sequence and resulting amino acid sequence do not offer additional insight into a potential role for the protein in the initiation of DNA replication. In fact, database comparison reveals only limited homology between *RAR1* and a protein which binds the poly(A) tail of mRNA. Perhaps the *RAR1* protein is a single-stranded-DNA-binding protein involved in DNA replication. The fact that temperature-arrested *rar1-1* cells do not have uniform bud sizes (as in the case for *cdc* mutants) suggests that the action of the *RAR1* gene product is not limited to S phase. Also while the *rar1-1* mutant improves the mitotic stability of a derivative containing a mutation outside the consensus sequence, it does not stabilize a derivative with mutations within the consensus. Thus, the *rar1-1* mutant lacks certain expected phenotypes of an initiator protein mutant.

One biochemical approach to isolating the initiator protein involves purification of proteins which interact specifically with *ARS* DNA sequences. Several laboratories have purified proteins which interact with *ARS* DNA as judged by DNase 1 footprinting and gel retardation assays [33,44-46]. Certain of the proteins appear to be identical, independent isolates, while others appear distinct. However, none of the proteins is likely to be the initiator protein since (1) none of the proteins isolated to date bind specifically to the core consensus sequence and (2) the isolated proteins interact with only a subset of the *ARS* elements tested for binding. These findings have led to the speculation that the identified proteins are accessory to the initiation complex and facilitate initiation at a specific subset of *ARS* elements. However, the significance of the protein binding sites in *ARS* function has been tested for only one protein, *ARS* binding factor I (ABF-I; [33,46]). The smallest

deletions into the footprinted region result in only minimal decreases in the efficiency of initiation of DNA replication [46]. Conclusive evidence of the significance of the isolated proteins as determinants for the initiation of replication would require point mutations which eliminate binding and reduce the efficiency of initiation but do not alter other properties such as the unwindability of the origin.

A slightly different biochemical approach utilizes a high molecular weight ($2 \cdot 10^6$) replication complex isolated from *S. cerevisiae* [47,48]. The complex binds tightly to *ARS*-containing sequences and does not discriminate between two different *ARS* elements tested for binding. The analysis of this complex has already provided suggestive evidence that the *CDC7* protein kinase is a component of the replicative complex [49]. Further dissection of this complex may lead to identification of the initiator protein.

V. A model for the initiation of DNA replication in *S. cerevisiae*

Based upon the sequence requirements for *ARS* function and the unwinding properties of yeast replication origins, Umek and Kowalski have proposed a specific mechanism for the initiation of DNA replication in *S. cerevisiae* (Fig. 3). By analogy to other well-studied replication systems, the authors presume that an initiator protein recognizes the core consensus sequence. The core consensus sequence is referred to as the 'initiator recognition element'. It is proposed that as a consequence of initiator protein recognition of the core consensus sequence, the *ARS* flanking sequence becomes locally unwound. Unwinding of the flanking sequence (melted as shown or possibly partially unwound) facilitates the entry of the replication complex, which is unable to enter B-form DNA. The flanking sequence required for efficient opening is termed the 'DNA unwinding element' (*DUE*). The efficiency of unwinding is, at least in part, determined by the primary sequence of the *DUE* and the ambient temperature, since both have been shown to contribute to the overall ease of origin unwinding (Ref. 3; Umek and Kowalski, unpublished data). This model is consistent both with the observation that *ARS* function requires a core consensus (protein recognition) sequence which is sensitive to single base mutations [25-28] and with the observation that *ARS* function is not affected by mutations in the *DUE* which do not significantly alter the energy requirement for unwinding the region [3].

The model presented in Fig. 3 borrows from mechanisms which have been shown to contribute to the initiation of DNA replication at certain well-studied prokaryotic replication origins [39,50,51]. Both *oriC* and the bacteriophage λ replication origin contain A + T-rich DNA sequences adjacent to their initiator binding

Fig. 3. A model for the initiation of DNA replication in *S. cerevisiae* proposed by R.M. Umek and D. Kowalski. See text for further details.

sites. It has been shown that these A + T-rich sequences are the sites of localized unwinding during the initiation of DNA replication in vitro. Localized unwinding results in sensitivity of the unwound region to a single-strand-specific nuclease [39,50,51]. The locally unwound sequences are thought to facilitate the entry of a DNA helicase which is unable to enter the B-form helix [52]. The authors of the *oriC* study have postulated that the A + T-rich region is unwound through direct interaction with the initiator protein [39]. However, it has been shown that the same region is hypersensitive to single-strand-specific nucleases in the absence of the initiator protein ([4]; Eddy, M.J. and Kowalski, D., unpublished results), suggesting that a low free energy requirement for helical unwinding alone may account for the localized unwinding of the A + T-rich region of *oriC*. The *DUE* of yeast *ARS* elements can also adopt a nuclease-hypersensitive conformation in the absence of replication proteins. This similarity suggests that the *DUE* of yeast origins may, like the locally unwound regions of the prokaryotic origins, serve as the entry site for a DNA helicase (Fig. 3). The similarity between yeast and prokaryotic origins also suggests that the *DUE* may be a component of replication origins which has been conserved throughout evolution.

The presented model suggests that the overall efficiency of initiation of yeast DNA replication would reflect the weighted contributions of the multiple components of an origin. We anticipate that the affinity of initiator protein binding to the core consensus sequence will prove to vary from *ARS* to *ARS*, since the core

consensus sequence itself varies. We further speculate that initiator binding affinity will prove to be a determinant of the efficiency of utilization of a given replication origin. In addition, the energy required to unwind the *DUE* should also affect the efficiency of initiation. As noted above, mutations which reduce the ease of origin unwinding in vitro result in temperature-dependent initiation in living cells. The efficiency of initiation is also temperature-dependent for certain wild-type *ARS* elements (Umek, R.M. and Kowalski, D., unpublished data) and for the origin in the yeast rDNA repeat (Linskens, M.H.K., unpublished data). These variable determinants of initiation efficiency may account for the observation that certain sequences appear to be 'weak' *ARS* elements, while others appear 'strong' in *ARS* activity assays. It is likely that weak *ARS* elements either bind the initiator protein weakly and/or require relatively greater energy to unwind their *DUE*. The model presented above suggests that both protein-DNA interactions and the energetics of the template are determinants of the frequency with which a given sequence acts as an origin of replication on the chromosome.

VI. Localization of higher eukaryotic replication origins

Numerous attempts to identify higher eukaryotic origins have been made during the past decade, and these studies have led to apparently conflicting conclusions. On the one hand, several types of experiment have suggested that no specific sequences are required

for origin function (see below); on the other hand, numerous experiments have suggested that origins are localized to preferred sequences (see further below), although none of these studies has provided sufficient resolution to allow determination of the characteristics of these preferred origin sequences. We shall briefly review the experiments leading to both types of conclusion and then suggest a way in which the apparent conflict might be resolved.

No specific sequence required for origin function?

The first suggestion that, under certain circumstances, replication could initiate without requirement for specific sequences came from an experiment in which *tsA* mutants of SV40 were incubated at restrictive temperature, thereby reducing viral DNA replication by 97% [53]. Electron microscopic analysis showed that at least half of the remaining replication was due to initiation at random sites around the genome, not at the SV40 origin [53]. One possible explanation of this observation is that, when the frequency of T-antigen-catalyzed initiation at the SV40 origin was sufficiently reduced by incubation at the restrictive temperature, a "background" of initiation due to the normal cellular replication machinery could be detected, and, perhaps, because the SV40 genome lacks a preferred origin site recognized by the normal cellular machinery, this "background" initiation did not exhibit sequence preference.

More direct evidence for initiation without sequence preference, again in a special situation, was provided by studies on the fate of circular DNA molecules injected into *Xenopus laevis* eggs [54]. Such molecules were found to replicate semiconservatively and in a regulated fashion: each molecule replicated no more than once per (pseudo) cell cycle. All tested circular molecules replicated with approximately equal efficiency; no requirement for a specific origin sequence was detected [54].

A fiber autoradiographic study of DNA replication in *Drosophila* polytene chromosomes has provided evidence that initiation sites are not absolutely specific [55]. Autoradiographic patterns suggestive of initiation were frequently misaligned, both temporally and spatially, in some of the multiple parallel chromatids of which polytene chromosomes are composed. These misalignments suggested that alternative origins could be used for replication of the same chromosomal regions [55].

Evidence for specific origin sequences

Numerous approaches have been used in attempts to identify origin sequences in higher eukaryotic cells. Al-

though earlier methods lacked resolution or relied on one or more untested assumption(s), several newer approaches suggest strongly that specific replication origins *can* be identified with confidence in higher eukaryotic cells. In the following discussion we attempt to distinguish the most promising current approaches from those that seem less likely to provide unambiguously interpretable results.

Electron microscopy. Although electron microscopy cannot be used to identify origins in the unique chromosomal DNA of eukaryotic cells, it can be used to map origins in highly repeated chromosomal sequences or in autonomously replicating elements. In fact, the most precisely localized eukaryotic replication origins are probably those in the rDNA repeat of sea urchins [56] and in the extrachromosomal palindromic rDNA of *Tetrahymena* [57,58] and *Physarum* [59], all mapped by electron microscopy. In all three cases (as in all other studied examples of rDNA replication), the origins are located in the nontranscribed spacer region upstream of the rDNA genes.

Earliest replication. One of the most straightforward approaches to identification of origins is offered by replication timing studies. Within a single replicon, the segments of DNA closest to the origin should replicate the earliest. An excellent example is provided by the mouse immunoglobulin heavy chain constant region gene cluster. In cells which do not synthesize immunoglobulins, the genes in this cluster replicate in the same order as their order in the chromosome, with the C_{α} gene replicating earliest and the D segments replicating last [60]. The distances between the genes, divided by the differences in their times of replication, suggest a fork movement rate of 1.7 to 1.9 kb/min [60], consistent with mammalian fork rates measured by fiber autoradiography [2]. These results suggest the presence of a replication origin just outside the gene cluster at the C_{α} end. An additional replication timing study [61] shows that two regions of the human β -globin locus, one near the γ -globin gene and the other about 18 kb downstream of the β -globin gene (the two regions are about 42 kb apart) appear to replicate slightly earlier than neighboring regions in K562 cells (in which the embryonic and fetal globin genes are transcribed). However, the entire locus replicates early in these cells, so the data do not yet distinguish between two specific origins located at the earliest replicating sites and multiple origins which fire at slightly different times [61]. A clearer picture was obtained in studies of the timing of replication of different portions of the dihydrofolate reductase (DHFR) gene region in CHO 400 cells, in which this gene region is amplified about 1000-fold [62,63]. The cleanest results, obtained with an in-gel renaturation procedure which eliminates background due to non-amplified sequences, suggest the existence of two origins about 22 kb apart, both downstream of the

DHFR gene. These origins were localized by timing studies to restriction fragments of 1.7 and 1.8 kb [63].

Strand extrusion. The short nascent strands expected to be located in small replication 'bubbles' surrounding newly-initiated origins can be extruded from those bubbles by the process of 'branch migration' at elevated temperatures [64]. Another method for isolating such short, origin-containing, nascent strands from small bubbles is to cross-link DNA in intact cells at 1–5 kb intervals with psoralen or a psoralen derivative, then allow cells to continue replicating DNA after cross-linking [65]. DNA synthesized at origins located between cross-links should be elongated only up to the cross-links (and thus remain short). The origin-containing nascent strands should not be cross-linked to parental strands and should be separable from parental strands by alkaline denaturation [65]. Although it seems likely that this cross-linking technique would allow identification of normal replication origins, it is also possible that the cell may respond to the cross-linking by induction of additional (abnormal) origins between cross-links [66]. For both strand extrusion techniques, subsequent extensive purification of the extruded strands should provide significant enrichment for origin sequences and the bulk properties of the extruded strands should reflect the bulk properties of origin sequences. However, if the extruded sequences are cloned [67], there is no way to determine (without using other methods) whether any individual clone corresponds to a real origin or is simply a consequence of contamination of the nascent strand preparation by non-origin DNA.

Despite this reservation, the strand extrusion techniques [64,65] can be used to determine whether specific DNA sequences are likely to be origins if the extruded strands are used as hybridization probes. Origin-containing restriction fragments should hybridize with the extruded strands to a greater extent than non-origin-containing fragments. This approach has been used to locate an apparent origin about 3 kb upstream of the α^E gene and a possibly weaker apparent origin between the α^D and α^A genes in the chicken α -globin domain [68]. The suggestion that an origin is located near the α^E gene is consistent with data obtained by *in vitro* runoff (see below). In addition, an apparent origin has been localized to 3.8–4.6 kb upstream of the transcription start site in the rat ribosomal DNA (rDNA) repeat unit [69], a location consistent with the position of the replication origin in the human rDNA repeat (Russev, G. and Huberman, J.A., unpublished data; see below). Furthermore, when this approach was used to investigate the location(s) of origins in the amplified DHFR DNA of CHO 400 cells, two apparent origins were found [70] which corresponded (within experimental error) to the two origins found by replication timing studies [63]. This agreement between results obtained with independent techniques tends to support the validity of each technique.

***In vitro* runoff.** An alternative approach to localization of origins and determination of direction(s) of replication is offered by the *in vitro* runoff (IVR) technique [71]. Nuclei are isolated from asynchronously growing cells, incubated with a restriction enzyme (restriction enzyme No. 1), and then allowed to continue DNA synthesis *in vitro* in the presence of BrdUTP. Subsequently, DNA is isolated, cut with an additional restriction enzyme(s) (restriction enzyme No. 2), and fractionated according to density by isopycnic centrifugation. Then the proportion of restriction fragments of interest which appear in heavy and light fractions is determined. If one assumes that *in vitro* DNA replication is simply a continuation of replication initiated *in vivo*, that no new initiations occur *in vitro*, and that *in vitro* replication proceeds smoothly, with the only barriers to replication being normal termination sites and the intentionally introduced restriction sites (No. 1), then BrdUTP should be preferentially incorporated into normal termination sites or into those ends of No. 1 restriction fragments towards which replication forks move. Data obtained with this technique are consistent with the existence of a replication origin 3–5 kb upstream of the α^E gene in the chicken α -globin locus ([71] and Berberich, S.J. and Leffak, M., unpublished data) in striking agreement with the results of a strand-extrusion investigation ([68]; see above). Additional studies using the IVR technique suggest that the chicken histone H5 gene is replicated from an upstream origin in tissues where this gene is active, and from a downstream origin in tissues where it is inactive [72]. Likewise, transcriptional status appears to be correlated with direction of replication through the *c-myc* locus, with an origin just upstream of the *c-myc* gene being active in HeLa cells where the gene is transcribed and inactive for the unarranged copy of the gene in Burkitt lymphoma cells, where the gene is not transcribed (Leffak, M. and James, C.D., submitted). This observation of a potential replication origin upstream of the *c-myc* gene is reinforced by the additional observation that the *c-myc* upstream region appears to permit autonomous replication of a plasmid in HeLa [73] and HL-60 [74] cells, just like an *ARS* element in yeast cells.

ARS elements in mammalian cells? After the discovery of *ARS* elements in yeast, numerous investigators attempted to detect similar elements in higher eukaryotic cells, especially in mammalian cells. Most of these investigations yielded negative results and were never published. A published example of such negative results is provided by reference [75].

However, many positive reports of *ARS* activity in mammalian cells have appeared recently. Several criteria are usually employed to demonstrate autonomous replication of transfected plasmids: (i) test for loss of bacterial and gain of mammalian methylation patterns by *DpnI* and *MboI* digestion; (ii) demonstration of semiconservative replication by density shift; and (iii)

demonstration that the transfected sequences remain as free plasmids by separation of cellular DNA into low- and high-molecular-weight fractions plus gel electrophoretic size analysis and blot hybridization. Despite the use of such criteria, in two cases, initially apparently positive results subsequently proved to have been misinterpreted ([76,77]; Grumati, F., personal communication). In yet another case, positive results reported by one laboratory [78] were not reproduced by a different laboratory [79]. And, with the exception noted in the previous paragraph, none of the positive results obtained so far (see next paragraph) has yet been reproduced independently. These problems suggest that, until independent confirmation has been obtained, existing positive reports of *ARS* activity in mammalian cells should be viewed with cautious skepticism. Even if some of the apparent *ARS* elements detected by various laboratories prove to be reproducible, a physical method for origin mapping must be used to test whether the *ARS* element functions as an origin in its chromosomal context; experience with yeast suggests that many *ARS* elements are likely to be inactive as chromosomal origins (see above). It will be fortunate if some of the reported *ARS* elements prove to be reproducible and to function as origins: if so, then *in vitro* mutagenesis could easily be used to determine at least some of the nucleotide sequence requirements for mammalian origin function, as has been done in yeast.

An encouraging correlation between physical techniques for mapping origins and the *ARS* assay is provided by the demonstration [80] that, of 12 cloned sequences selected as possible origins by a strand extrusion technique [64], 4 seemed to permit at least transient autonomous plasmid replication. Especially exciting, if true, are the recent demonstrations by the Ariga group that three nuclear oncogenes, *c-myc* [74,78], *N-myc* (Ariga, H., personal communication) and *p-53* [81], all appear to bind to specific DNA sequence elements (different for each oncogene), and that these DNA sequence elements can serve as efficient *ARS* elements in cells expressing the appropriate oncogene!

The reality of the phenomenon of autonomous replication has recently obtained independent support from the demonstration that mammalian cells in the process of amplifying genes sometimes contain large (120–750 kb) closed circular DNA molecules which replicate semiconservatively approximately once per cell cycle ([82,83]; Ruiz, J.C., Choi, K., Von Hoff, D.D., Roninson, I.B. and Wahl, G.M., unpublished data). The ability of such large DNA molecules to replicate autonomously appears to depend on specific (not yet well characterized) sequences or sequence arrangements [82].

2D gel methods. Although the 2D methods described above were originally developed for analysis of yeast DNA replication, they are capable, without significant additional modification, of mapping replicons in the

unique chromosomal DNA of organisms with genomes as much as 10-fold more complex than yeast (such as *Drosophila*) and of mapping replicons in highly repeated stretches of chromosomal DNA in mammalian cells. Since the cells need not be synchronized or manipulated in other unnatural ways, the results provided by these 2D gel techniques are likely to permit unambiguous interpretation.

2D gel technology has already provided some interesting insights into the process of chorion gene amplification in *Drosophila*. In the follicle cells of this organism, the genes encoding chorion (eggshell) proteins are preferentially replicated during the final hours of oogenesis, apparently in order to provide sufficient template for the burst of chorion mRNA synthesis which occurs at that time. This amplification occurs by repeated initiation at sites near the two clusters of chorion genes, one on the X chromosome and the other on chromosome 3. P-element-mediated transformation has allowed identification of one or more *cis*-regulatory 'amplification control elements' and several modulating elements in each cluster, located close to or at the sites where replication initiates [84,85]. Because the replication forks responsible for this amplification move unusually slowly (50–100 bp/min [86]), a high proportion of the DNA restriction fragments isolated from these regions during late oogenesis contain replication forks, providing large signals for analysis by neutral/neutral 2D gel electrophoresis [10]. Results obtained so far suggest that 2 to 4 closely spaced origins are located within an interval of 1.5 to about 7 kb distant from the previously detected 'amplification control element' at the chromosome 3 cluster (Spradling, A.C., personal communication). Further genetic and physical analysis should provide improved resolution of the sequences required for control and origin function during amplification and should tell us whether the sites used as origins during amplification are also used as origins during normal S phase.

Russev and Huberman (preliminary observations) used a modified neutral/alkaline 2D gel method [12] to examine replication in human (HeLa cell) DNA. Not surprisingly, they detected an origin just upstream of the 45 S transcription unit. More surprising was the observation that replication appeared to proceed unidirectionally through the transcription unit and then on through the approx. 30 kb non-transcribed spacer, all in the direction of transcription.

How specific are origins?

The available evidence (reviewed in Section II above) suggests that initiation of yeast DNA replication is a stochastic process, with preferred origin sequences 'firing' at origin-specific frequencies varying from 100% (once per S phase in all cells) down to less (Linskens,

M.H.K. and Huberman, J.A., unpublished data) than the detectability limit of about 10% (once per S phase in 1 out of 10 cells) [13]. None of the studies of origin usage in higher eukaryotic organisms has yet provided a frequency estimate, but the fact that non-specific origin usage is detected in some circumstances (reviewed above) while at least partially specific origin usage is detected in others (reviewed above) suggests that initiation in higher organisms may also be stochastic, with the frequency of origin usage being controlled by the concentrations of a variety of *trans*-acting factors important for establishing an appropriate chromatin structure and for initiating replication. It is likely that the *Xenopus* egg contains an extremely high concentration of such factors, allowing virtually any sequence to function as an origin [54]. In normal somatic tissues, the concentrations of (some of) these factors is probably lower, allowing initiation primarily only at the sequences to which the limiting factors preferentially bind. The implication that different factors may be limiting in different tissues (giving rise to tissue-specific origin usage) is consistent with the observations cited above which suggest that certain genes are replicated in the transcriptional direction when they are being transcribed, but in the counter-transcriptional direction when they are repressed ([72]; Leffak, M. and James, C.D., unpublished data) and with the observation that certain sequences function as *ARS* elements only when the cell is expressing certain oncogenes ([74,81]; Ariga, H., personal communication).

VII. Regulation of replication

Each chromosomal DNA molecule is normally replicated once and only once during a single S phase. What are the signals which lead to initiation of replication during S phase, and what are the mechanisms that prevent replicated DNA from initiating again during the same S phase? Partial answers to both questions are suggested by recent studies on the regulated replication of sperm chromatin introduced into *Xenopus* egg extracts [87–89]. Such chromatin must be assembled by the extract into nuclei with intact nuclear envelopes before the sperm DNA can replicate [88]. Once replication is initiated, the DNA in each nucleus is replicated completely. The reconstituted nuclei are also capable of a second round of DNA synthesis, but only after the nuclear membrane is permeabilized [89]. Normally this permeabilization of the nuclear membrane takes place when the reconstituted nuclei in egg extract undergo a series of mitotic-like events, including nuclear envelope breakdown, after their first round of DNA synthesis. Even in the absence of mitosis, however, permeabilization of the nuclear membranes by mechanical shear or by lysolethycin treatment permits an additional round of DNA synthesis [89]. These observa-

tions suggest that replication might be controlled by a cytoplasmic 'Licensing Factor' which could normally gain access to the nuclear DNA only during mitosis, permitting a subsequent single round of DNA synthesis [89].

The suggestion [89] that 'Licensing Factor' binds to DNA when the nuclear membrane is permeabilized during mitosis is an attractive one, but it fails to fully explain the observation [89] that, under conditions where nuclei are non-optimally permeabilized (e.g. by lysolethycin) only a fraction of the nuclei re-replicate, and each nucleus re-replicates either fully or not at all. This all-or-none response suggests that 'Licensing Factor' may be involved in cooperative interactions; perhaps these interactions involve nuclear structure and not simply nuclear DNA. Whatever the mechanism, the data suggest that the intranuclear concentration of 'Licensing Factor' must exceed a certain threshold level for initiation to proceed.

Earlier experiments have shown [54] that limitation of replication in *Xenopus* eggs to a single round per (pseudo) cell cycle does not require any specific DNA sequence. Thus, the type of *cis*-acting sequence required by bovine papilloma virus to limit replication to one round per cell cycle [90,91] appears to play no role in the limitation of *Xenopus* egg DNA synthesis to one round. An additional argument against a role for *cis*-acting sequences comes from the studies of the DNA sequence requirements for *ARS* element function in yeast (reviewed above): the two *cis*-acting elements identified (the *ARS* consensus sequence and the *DUE*) are essential for *initiation* of replication but do not appear to affect the limitation of replication to one round per S phase. In fact, to our knowledge, no mutations of *ARS* elements leading to overreplication have yet been identified, suggesting that restriction of replication to a single round per S phase is not controlled by specific *cis*-acting DNA sequences.

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