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S. E. Kearsey

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Initiation of DNA replication in yeast chromosomes

By S. E. Kearsey

Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, U.K.

A family of DNA fragments from the yeast genome has properties that suggest that chromosome replication starts at specific DNA sequences. These elements (autonomously replicating sequences: ARS) have a bipartite structure: a small (less than 20 base pairs) AT-rich region essential for function, flanked by larger regions important for maximal activity of the replicator. In an attempt to identify proteins involved in initiation of replication, yeast mutants that show an enhanced ability to replicate minichromosomes with defective ARSs have been isolated.

Location of replication origins in eukaryotic chromosomes

The initiation of DNA replication in prokaryotic chromosomes appears to be the crucial step for the regulation of the entire process. This event generally involves the interaction of initiator proteins with a specific DNA sequence constituting the origin of replication. Although replication origins are well characterized in prokaryotic chromosomes, less is known about the multiple initiation sites in eukaryotic DNA. The indications are, however, that eukaryotes resemble prokaryotes in that DNA replication starts at specific sites. Attempts to locate initiation sites in eukaryotic DNA have had most success with origins that are repeated many times in the genome, because this makes it easier to detect an event that only occurs once per cell cycle at a single origin. Botchan & Dayton (1982) used electron microscopy to examine the location of replication bubbles in purified rRNA genes from *Lytechinus*, and showed that initiation occurs in a specific region of the non-transcribed spacer, 3′ to the 26*S* gene. Heintz *et al.* (1983) showed that restriction fragments from the highly repeated *DHFR* gene in a methotrexate-resistant hamster cell-line show a temporal order of replication, consistent with the notion that replication starts from a particular site in the repeated unit. Replication origins in eukaryotic viruses such as SV40 and adenovirus are of course well studied, but because they function to uncouple replication of the viral genome from the cell-cycle control exerted by the host cell, they may not be typical of chromosomal origins.

Further evidence for the initiation of eukaryotic replication at specific sequences comes from the yeast *Saccharomyces*. DNA molecules introduced into yeast cells by transformation cannot replicate autonomously unless they contain chromosomal sequences called ARS (autonomously replicating sequence) elements, which may be providing a necessary replication origin (for review see Kearsey (1986)). Consistent with this interpretation is the observation that replication bubbles in the *Saccharomyces* rRNA gene repeat originate from a region containing an ARS (Saffer & Miller 1986). Also, it is clear that the replication associated with ARSs is mediated by the same mechanism that is responsible for the replication of the nuclear DNA.

Although this evidence suggests that replication origins are specified similarly in both prokaryotic and eukaryotic chromosomes, there are also findings which suggest that eukaryotes

can change the distribution of replication origins in chromosomes under special conditions. A dramatic demonstration of this is provided in the cleavage nuclei of *Drosophila*, where a very short S-phase is achieved by a close spacing of replication origins (Blumenthal *et al.* 1974). The much longer S-phase of *Drosophila* cultured cells is mirrored by a longer distance between adjacent replication origins; it is unclear how this change in spacing is brought about. A related finding is that the *Xenopus* egg will replicate any DNA that is microinjected into it without apparent sequence specificity and with cell cycle control (Harland & Laskey 1980; Méchali & Kearsey 1984). Perhaps the short S-phases in early *Xenopus* development result from a lack of stringent sequence requirements for initiation of replication?

The use of multiple initiation sites is clearly an adaptation allowing the replication of eukaryotic chromosomes (average size 500–1000 kilobases in *Saccharomyces*) in a relatively short S-phase (about 20 min in *Saccharomyces*) with a elongation rate of only about 3 kilobases per minute. Because the length of S-phase depends on the distribution of initiation sites, there may be selective pressures on cells to change their replication origin distribution in order to achieve faster cell division rates. Such changes might evolve via mutations in the DNA sequence of the chromosome to produce a more frequent occurrence of specific replication origins. However, the observations made in *Drosophila* and *Xenopus* suggest a more global solution, that of lowering the stringency of the sequence requirements for initiation, so that appropriate sites occur frequently enough by chance. Perhaps both mechanisms contribute to changed replication origin spacing? Genetic selection for specific origins by sequence changes might be especially important in regions of the chromosome that show low sequence complexity or if origin location affects other genetic processes such as transcription control or chromosome behaviour in mitosis. With regard to this last point, Sundin & Varshavsky (1981) have proposed that collision between replication forks may result in the intertwining of daughter DNA molecules owing to the steric exclusion of DNA topoisomerase; this intertwining may explain pairing of sister chromatids in metaphase. Murray & Szostak (1985) have emphasized the importance of an appropriate distribution of replication origins to achieve convergence of replication forks. For instance, a linear chromosome with a single origin should not become intertwined during replication and might therefore show a high frequency of nondisjunction. These considerations may explain why the subtelomeric regions of yeast chromosomes contain multiple ARSs (Chan & Tye 1983), which could serve to drive replication forks into both ends of the chromosome and thus ensure intertwining irrespective of the internal distribution of replication origins.

Our understanding of the initiation of DNA replication is poor because few of the eukaryotic proteins that are involved in the process have been identified. I have been using genetic methods in an attempt to identify replication proteins in yeast. One approach has been to characterize sequences that support autonomous replication in the yeast cell, and to isolate mutants where the activity of ARS elements is affected.

Sequence requirements for autonomous replication in yeast

Transformation of *Saccharomyces cerevisiae* cells with plasmids provides a qualitative assay for ARS activity. ARS-containing (Ars^+) plasmids transform yeast cells much better than Ars^- plasmids, presumably because they do not have to integrate into an endogenous chromosome, which represents an inefficient step. The situation is rather different in the fission yeast, *Schizosaccharomyces pombe*. Here, Ars^- plasmids may transform with moderate efficiency, but

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examination of the transformants shows that the plasmid is not replicating as a simple monomer, but is either integrated into a chromosome, or grossly rearranged (Maundrell *et al.* 1985).

Although transformation frequencies in *Saccharomyces* can give a qualitative index of ARS activity, the analysis of the mitotic stability of centromere-containing plasmids shows clearly that the capacity of a sequence to confer autonomous replication is a quantitative property. Plasmids containing yeast centromeres alone do not function as minichromosomes, but also require ARS elements. Different ARS elements confer different mitotic stabilities on centric plasmids, and mutations in an ARS may reduce the mitotic stability that it confers on a centric plasmid without completely abolishing replication (Murray & Szostak 1983; Koshland *et al.* 1985) (table 1). ARS mutations reduce the replication, but not the segregation efficiency of centric plasmids as expected and plasmid loss by degradation does not occur. These observations imply that ARSs are continuously required for plasmid replication, and are not just transiently needed during transformation. The efficiency of ARS function may correlate with the probability that the sequence will fire as an origin of replication in a particular S-phase.

TABLE 1. MITOTIC LOSS RATES OF *CEN3*-CONTAINING PLASMIDS

ARS†	yeast strain	loss rate‡
ARS1	R609-20-1 (wt)	0.8
ARS1	W303-1A (wt)	0.06
HO ARS§	R609-20-1 (wt)	0.24
ARS1+*HO* ARS	W303-1B (wt)	0.04
ARS1	R179-1 (Rar$^-$)	0.04
HO ARS	R179-1 (Rar$^-$)	0.04
HO ARS	R193-8 (Rar$^-$)	0.12
ARS1	R193-8 (Rar$^-$)	0.04
HO ARS	R411-6C¶ (Rar$^-$)	0.15

Strains are described in Kearsey (1987).

† With strains derived by mutagenesis, original plasmid was eliminated by curing, and fresh plasmid was introduced by transformation. All plasmids contained *CEN3* and *SUP4-o*.

‡ Plasmid-containing cells were grown into log-phase in non-selective medium, then sonicated and plated out onto non-selective plates. The value given shows the frequency of half-sectored colonies, where the first cell division resulted in the production of a cell not containing the plasmid, as a fraction of the total number of colonies derived from plasmid-containing cells (see Hieter *et al.* 1985).

§ The *HO* ARS used was the 1062 allele (figure 2) (Kearsey 1984).

¶ Derived from the 339-13 mutant (table 2).

Qualitative assays, involving the efficiency of yeast transformation, and quantitative assays, measuring the mitotic stability of centric plasmids have been used to define the sequence requirements for ARS activity. Three ARS elements have been analysed in detail and a consistent picture of ARS structure has emerged (figure 1). First, ARS elements contain a small AT-rich region of 13–19 base pairs (b.p.) that is essential for autonomous replication (defined here as the E-domain). The E-domains of different ARSs show obvious sequence homology. Deletions into this region abolish autonomous replication, and point or linker mutagenesis of this sequence can also eliminate ARS function. Second, the essential region is flanked by a sequence that is important for autonomous replication (defined here as the I-domain). Deletions in this I-domain reduce, but may not eliminate, replication. The apparent extent and location of the I-domain is dependent on the assays used for ARS function. With a semi-quantitative

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transformation assay, the I-domain of the *HO* ARS was shown to be only about 20 b.p. 3′ to the T-rich strand of the E-domain (Kearsey 1984). With more sensitive assays based on the mitotic stability of centric plasmids, the I-domain is considerably larger. In the case of *ARS1*, a region of about 130 b.p. is required 3′ to the E-domain T-strand (see figure 1); in addition, a region of 200–300 b.p. on the other side of the E-domain has a detectable, although smaller, effect on autonomous replication (Celniker *et al.* 1984; Koshland *et al.* 1985; Synder *et al.* 1986). This bipartite structure of ARS elements is reminiscent of a number of viral replication origins which have both essential and important regions.

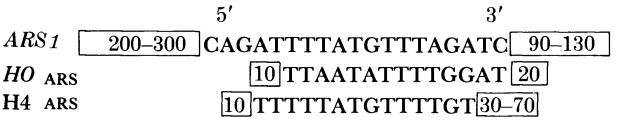

FIGURE 1. ARS structure. The figure compares the essential DNA sequences (E-domains) of *ARS1*, the *HO*-linked ARS and the H4-linked ARS. The boxes flanking the sequences represent DNA sequences important for autonomous replication (I-domains), and the numbers in the boxes represent the approximate extents of these regions in base pairs (not drawn to scale). The E-domains were defined by deletion analysis in the cases of *ARS1* (Celniker *et al.* 1984; Srienc *et al.* 1985) and the *HO* ARS (Kearsey 1984), and by insertional mutagenesis in the case of the H4 ARS (Bouton & Smith 1986). This minimal sequence of *ARS1* has been shown to be sufficient for weak ARS function in some sequence environments (Srienc *et al.* 1985). The extent of the I-domain of the *HO* ARS was determined by measuring the growth rates of acentric plasmids (Kearsey 1984). In the case of *ARS1*, the extent of the I-domain has been determined by assaying the mitotic stability of centric plasmids which is a more sensitive assay for ARS function and probably partly explains the difference in the I-domain size between the *HO* ARS and *ARS1* (Koshland *et al.* 1985; Synder *et al.* 1986). The I-domain of the H4 ARS was defined by deletions which apparently abolish autonomous replication (Bouton & Smith 1986); the effect of these deletions is to some extent dependent on what new sequence is introduced. However, insertion of oligonucleotide linkers into the I-domain does not affect ARS function.

PROTEINS INVOLVED IN INITIATION OF REPLICATION

The identification of DNA sequences that are implicated in replication initiation provides reagents for the biochemical isolation of proteins involved in ARS function. In addition, by using genetic screens which monitor ARS activity, it may be possible to identify genes whose products function in replication initiation. For instance, Maine *et al.* (1984) have identified a number of Mcm^- mutants that show a reduced capacity to replicate certain Ars^+ plasmids. Another approach is to start with minichromosomes whose replication is driven by a defective ARS, and to isolate second-site suppressor mutations that restore the ability of the plasmid to replicate. Such mutations might elevate the concentration of replicator proteins, or qualitatively change initiator protein structure so that mutant ARS sequences can function (see Dotto & Zinder (1984)). Alternatively, mutations that reduce the function of hypothetical specificity factors, which normally restrict initiation events to specific origins, would effectively suppress defective ARS function (see Kaguni & Kornberg (1984) for a discussion of specificity factors in *E. coli* DNA replication). This second approach is described here, and some preliminary results are reported.

MUTATIONS THAT ENHANCE THE MITOTIC STABILITY OF MINICHROMOSOMES

The plasmid pSE276-1 contains a centromere (*CEN3*), a selectable marker (*URA3*) and a weak ARS derived from the *HO* element (figure 2) (Kearsey 1984, 1987). This plasmid is mitotically unstable, and its stability can be increased by the introduction of another ARS in *cis*

implying that it is replication rather than segregation deficient (table 1). The *HO* ARS element included in the plasmid contains two sequence changes that reduce its ARS activity compared with the wild-type ARS. The plasmid also contains a tRNA gene (*SUP4-o*) that can suppress the *ade2-1* mutation and on appropriate media the effect of this suppression is to make red *ade2-1* cells white. On rich media, the plasmid is rapidly lost, and this can be seen by the rapid sectoring of originally white colonies to red. This screen allows detection of mutations which improve plasmid stability; such mutants will show a reduced rate of sectoring (see Hieter *et al.* (1985) and Koshland *et al.* (1985) for a description of similar systems).

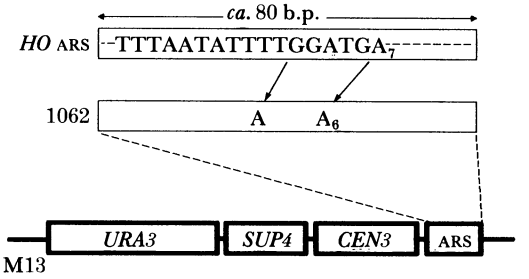

FIGURE 2. Structure of pSE276-1. This minichromosome was constructed by inserting the yeast *URA3* and *SUP4-o* genes into phage M13mp9 (Kearsey 1987). The plasmid also contains a restriction fragment containing a centromere (*CEN3*) and a weak ARS, and a mutant derivative of the *HO* ARS which has weak ARS function (this is the 1062 allele described in Kearsey (1984)). Sequence differences between the wild-type *HO* ARS and the derivative used in this plasmid are shown. Although pSE276-1 shows a mitotic loss rate that is greater than a similar plasmid containing the wild-type *HO* ARS, other factors may contribute to the inefficiency of ARS function such as position effects caused by flanking DNA sequences and the proximity of the centromere (S. E. Kearsey, unpublished results).

Haploid yeast strains containing the pSE276-1 plasmid, or related plasmids, were mutagenized with ethyl methanesulphonate; this mutagenesis was generally followed by selection for the *URA3* marker on the plasmid to enrich for mutants with the desired phenotype. About 10000 cells were then plated out onto non-selective media and this allowed screening for colonies whose sectoring suggested an improved mitotic stability of the minichromosome (described here as a Rar$^-$ phenotype). Mutants chosen for study (table 2) show similar growth rates irrespective of whether the plasmid is present or not, implying that the changed colony appearance is not a simple consequence of changed relative growth rates.

To determine the location of the mutations causing the Rar$^-$ phenotype, the mutants were cured of their original plasmids and retransformed with new DNA. All the mutants still showed improved mitotic stability of the minichromosome, showing that the mutation is not plasmid-borne but maps to the yeast genome. The mitotic stability of the pSE276-1 plasmid in the mutants was measured by plating out plasmid-bearing cells onto non-selective media and determining the frequency of plasmid-containing colonies that were half-sectored. Such colonies are produced if the first cell division of the yeast cell on the plate produces a plasmid-deficient cell (table 1). The original strain shows about 24% of half-sectored colonies, whereas in the best mutant 179-1, this is reduced to 4%. None of the Rar$^-$ mutants permit the replication of the Ars$^-$ plasmid YIp5, but it is unclear as yet whether the Rar$^-$ mutants can improve the stability of any weak ARS plasmid or whether the effect is specific to the original pSE276-1 plasmid.

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TABLE 2. Rar⁻ MUTANTS SHOWING ENHANCED MITOTIC STABILITY OF 'WEAK ARS' PLASMIDS

mutant†	loss rate, wt/mutant for pSE276-1‡	complementation group	other phenotypes§
179-1	6	?, partly dominant	SPD
193-8	2	2	—
199-2	nd	2	—
199-3	nd	4	—
200-2	nd	4	—
199-1	nd	3	—
340-5	nd	3	—
339-13	1.6	1¶	SPR, TS

Complementation data is taken from Kearsey (1987).

† Mutants in the same complementation group were derived from different mutagenesis experiments.

‡ Mutants were cured of plasmid, retransformed with fresh pSE276-1, and loss rates were determined as described in table 1.

§ SPD stands for dominant germination mutant; SPR stands for recessive sporulation mutant; TS represents temperature sensitive; cells arrest randomly in the cell cycle at 36 °C.

¶ Mutation (*rar1*) maps to chromosome 13R, near *rna1* (Kearsey & Edwards 1987).

Genetic characterization of the mutations shows that they are usually recessive, although the phenotype of 179-1 is partly dominant. Complementation analysis between the eight Rar⁻ mutants shown in table 2 defined four complementation groups (data not shown). Because the phenotype under study is quantitative, the number of complementation groups that can be defined by using this screen is dependent on what degree of improvement in plasmid stability is deemed to be required for the mutant to be described as Rar⁻. Thus although complementation analysis between a small number of mutants defines a relatively large number of genes, this is to some extent a reflection of the weak Rar⁻ phenotype of some of the mutants. Some of the Rar⁻ mutants show additional phenotypes that may be caused by the same mutation that is responsible for the Rar⁻ phenotype. This is the case with the 339-13 mutant, where the Rar⁻ mutation also prevents growth of the cell at 36 °C, and this temperature-sensitivity has made it straightforward to map the gene (table 2) (Kearsey & Edwards 1987).

CONCLUSIONS

Replication origins in eukaryotic chromosomes appear to be specified by specific nucleotide sequences, but there is also evidence that under some conditions the spacing between origins can be changed, perhaps as a result of reduced sequence requirements for initiation. In yeast, which appears to show stringent sequence requirements for initiation, mutations can be isolated that improve the mitotic stability of plasmids containing weak replicators. At present, it is only possible to speculate about the mechanism whereby the Rar⁻ mutations improve plasmid stability. Most of the Rar⁻ mutations are recessive, have a quantitative rather than qualitative effect on the replication of defective ARS plasmids, and were isolated from a screen of only about 10000 mutagenized colonies. Perhaps the enhanced mitotic stability of weak ARS plasmids can be brought about by quantitative changes in the activities of specific proteins. Although a small panel of Rar⁻ mutants identifies a comparatively large number of genes, an obvious criterion for deciding which mutations are likely to have a direct involvement in ARS function is to select those that have a dramatic effect on autonomous replication.

It will be interesting to determine the origin to origin spacing and S-phase length in Rar⁻

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mutants, to see if these are reduced. Because the yeast genome contains a number of naturally weak ARSs, which perhaps generally have a low probability of functioning in a particular S-phase, it is possible that the number of origins firing in a particular S-phase would be increased. It will also be of interest to see whether any of the Rar$^-$ mutants gives clues as to how early embryos of *Drosophila* and *Xenopus* achieve such short S-phases.

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