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RESEARCH ARTICLE

A Review of DNA Replication Studies and Contemporary Researches in Yeast

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ABSTRACT

The DNA replication is very interesting phenomenon, which was deeply investigated in the past century soon after the discovery of DNA and its helical structure. Initially, the phenomenon was explored in the virus (SV40, Polyoma virus, Papilloma virus, Adenovirus, etc.), bacteria i.e. *E. coli* and later on, in more complicated organism i.e. higher bacteria, fungus, plant, animals to some extant etc. The DNA replication process and mechanism was best studied in the yeasts, budding yeast-*S. cerevisiae* and Fission yeast-*S. pombe* as a great deal of information is known in these two organisms. The genome wide studies in these two organisms provided a great deal of information regarding the possible initiation sites through out the genome but the actual applicability of the data needs to be determined experimentally.

INTRODUCTION

The DNA was proven as the genetic material and double helical structure was proposed in 1953 (Hershey and Chase 1952, Watson and Crick 1953). Soon after, the mechanism of duplication of the genetic material, termed as DNA replication, was explored within a short stretch of time, S phase, before cell division (cytokinesis) during every cell cycle in all the living organisms. DNA replication is fundamental to life, which is strictly regulated occurring once per cell cycle (reviewed in Legouras *et al.,* 2006) and preserves the genetic information by faithfully transmitting it through generations. Replicon model was proposed to explain DNA replication process in *E. coli* genome where DNA replication starts at specific *cis*-acting element, called replicator, with the help of *trans*-acting regulatory proteins, called initiators and replicon was defined as a genetic element that replicated from a single origin of replication (Jacob *et al.,* 1963). This model explains replication process in *E. coli* (Bramhill and Kornberg 1988) but it could not explain replication in more complicated organisms. In viral and prokaryotic genomes, replication initiates at a single, non-redundant, well-defined replicator or origin of replication (Kornberg and Baker 1992). In eukaryotic genomes, replication starts at multiple initiation sites/origins throughout the length of the chromosome during S phase of the cell cycle (Huberman and Riggs 1968). A number of origins are well defined now i.e. bacteria (*Ori*C of *E. coli*, origin of *Bacillus subtilis*), viruses (SV40 origin, Polyoma virus origin, Papilloma virus origin, Adenovirus origin, Herpesvirus origin, Epstein-Barr virus origin, Pox virus origin, Hepatitis virus origin, Geminivirus origin and Baculovirus origin) (reviewed in DePamphilis 1996) and prokaryotes (*Tetrahymena*; Cech and Brehm 1981, *Entamoeba histolytica*; Ghosh *et al,.* 2003) etc. The regulatory proteins for these origins and their role in replication have already been elucidated to a great extent (Kornberg and Baker 1992; DePamphilis 1996). Among the eukaryotes, origins are best characterized in the budding yeast, *Saccharomyces cerevisiae* in which small, specific DNA sequences ranging from ~100 to150 bp in length, known as Autonomously Replicating Sequence (ARS) elements (reviewed in Campbell and Newlon 1991) function as replication origins. Initially, the ARS elements were isolated on the basis of their ability to transform *S. cerevisiae* cells with high frequency when present in a plasmid presumably by functioning as replication origins in yeast cells (Stinchcomb *et al.,* 1979; Hsiao and Carbon 1979). Later, two dimensional gel electrophoresis origin mapping techniques (2D techniques) made it possible to physically map initiation sites within or very close to the ARS element in two yeast plasmids, *ARS1* plasmid and 2µ plasmid (Brewer and Fangman 1987; Huberman *et al.,* 1987). These 2D techniques are capable of differentiating the replicating molecules from non-replicating molecules topologically or by the sizes of the nascent strands. In 1988, the first yeast chromosomal origins were mapped to *ARS305* on chromosome III (Huberman *et al.* 1988) and to the ARS elements associated with the rDNA repeats (Brewer and Fangman 1988; Linskens and Huberman 1988). This was followed by a number of origin mapping studies aimed to map origins across long stretches of chromosomal DNA (Dubey *et al.,* 1991; Newlon *et al.,* 1991, 1993) and in the whole chromosome (Yamashita *et al.,* 1997; Friedman *et al.,* 1997; Poloumienko *et al.,* 2001). The results from these studies made it clear that all the chromosomal origins investigated in *S. cerevisiae* correspond to ARS elements but all ARS elements do not function as origins in their normal chromosomal context.

Genetic and mutational analysis has revealed that *S. cerevisiae* ARS elements contain three functional domains, namely, domain A, domain B and domain C. The domain A contains a close match of the conserved 11 bp ARS Consensus Sequence (ACS) and is essential for origin activity. Domain A is flanked by A+T rich domain B at the 3′ end of its T rich strand. It is divided into three sub-domains B1, B2, & B3. The domain A and any two of B1, B2, & B3 are required for the origin activity. Domain C present at the 5′ end of the core ACS sequence of some ARS elements is stimulatory in nature (Marahrens and Stillman 1992, 1994; Rao *et al.,* 1994; Theis and Newlon 1994). The conserved domain A was considered to be a putative protein binding site by analogy to the prokaryotic replication origins. Indeed, protein binding studies demonstrated that a complex of six proteins, the origin recognition complex, ORC, binds with domain A of all the

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tested ARS elements and plays an important role in the initiation of DNA synthesis (Bell and Stillman 1992; reviewed by Rowley *et al.,* 1994; Fox *et al.,* 1995). The ORC homologs have been found in a wide variety of organisms including fission yeast, *Drosophila*, *Xenopus*, Mouse and Humans (reviewed in Dutta and Bell 1997; Bell 2002). Now all the replication factors, which bind to ORC in a cell cycle specific manner, are well explored in *S. cerevisiae* because the replication origins were well defined in this organism (reviewed in Kelly and Brown 2000). Replication origins have also been mapped in other organisms including other yeasts like *Yarrowia lipolytica*, *Hansenula polymorpha*, *Pichia*, *Ustilago* and *Schizosaccharomyces pombe* (reviewed in Huberman 1999), the slime mould, *Physarum polycephalum* (Benard *et al.,* 1995; Pierron *et al.,* 1999), the protozoan parasite, *Entamoeba histolytica* (Ghosh *et al.* 2003), plants like *Pisum sativum* (Van't Hof and Lamm 1991), Drosophila (Delidakis and Kafatos 1989) and mammalian cells (Kitsberg *et al.,* 1993). Replication in *Xenopus* egg nucleus (Harland and Laskey 1980; Mechali and Kearsey 1984) and in animal cells, for example, chorion genes in *Drosophila* (reviewed in Orr-Weaver 1991), dihydrofolate reductase gene in CHO cells (Handeli *et al.* 1989), Lamin B2 (Giacca *et al.,* 1994) and human β-globin gene domain (Kitsberg *et al.,* 1993), all suggest that initiation sites are distributed in broad regions of several kilobase-pairs or larger called initiation zones (reviewed in Burhans and Huberman 1994).

Micro injection of DNA fragment into the nucleus of *Xenopus* eggs, showed random replication initiation without the requirement of specific sequences or elements (Harland and Laskey 1980); (Mechali and Kearsey 1984) although the replication of injected fragment was tightly coupled with the cell cycle as it was replicated once per cell cycle (Harland and Laskey 1980) suggested that replication starts non specifically. During the last decade, a number of microarray-based genome-wide origin analysis studies in budding yeast have resulted into the identification of nearly 400 or more potential origins throughout the yeast genome (Raghuraman *et al.,* 2001; Wyrick *et al.,* 2001; Yabuki *et al.* 2002; Feng *et al.* 2006; Nieduszynski *et al.* 2006; Xu *et al.,* 2006). Different genome wide origin mapping studies done during the past ten years have estimated the presence of ~300 to >1000 potential origins in *S. pombe* (Segurado *et al.* 2003; Dai *et al.* 2005; Feng *et al.* 2006; Heichinger *et al.,* 2006; Hayashi *et al.,* 2007; Mickle *et al.,* 2007; Kiang *et al.*, 2010; Hayano *et al.,* 2012).

Fibre autoradiography studies

DNA fibre autoradiography of mammalian cells, plant cells and several other cell types including budding yeast clearly demonstrated that replication initiates at multiple points called origins distributed along the chromosomal DNA as opposed to the single initiation sites in prokaryotic genomes (Huberman and Riggs, 1968; Callan 1972; Blumenthal *et al.* 1973; Stubblefield 1975; Hand 1975; Taylor and Hozier 1976; Van't Hof *et al.* 1978; Rivin and Fangman 1980; Dubey and Raman 1987; reviewed in Berezney *et al.* 2000, Bryant *et al.* 2001). The results from these studies revealed that: (i) the inter-origin distances are highly variable ranging from 15-300 kb with an average of approximately 100 kb, (ii) most of the replication forks are bi-directional, which usually occur in divergent pairs and the rate of fork migration is also highly variable ranging from 0.3 kb/fork/minute to 6.0 kb/fork/minute with an average of 3.0 kb/fork/minute, a rate significantly lower than that of prokaryotes where the forks grow speedily at a rate of ~1kb/fork/second (Mirkin and Mirkin 2005), and (iii) replication initiates simultaneously at several (5-10) adjacent origins suggesting their clustering and coordinated regulation. The latter finding was also strongly supported by a number of cytological and microscopic studies (Nakayasu and Berezney 1989; reviewed by Berezney *et al.* 2000). Thus, it can be estimated that a typical mammalian genome will have approximately 20,000 to 50,000 replication origins distributed throughout the genome. DNA fibre autoradiographic studies were not able to figure out whether initiation of replication occurred randomly or at specific nucleotide sequences.

Replication studies in budding yeast

The yeast transformation experiments done during late seventies provided the first indication that replication initiation could be sequence specific. Taking the advantage of yeast transformation technique (Hinnen *et al.,* 1978; Beggs 1978), small DNA fragments were isolated from budding yeast that was capable of transforming yeast cells at high frequency (Stinchcomb *et al.,* 1979; Struhl *et al.,* 1979; Hsiao and Carbon 1979; reviewed by Campbell and Newlon 1991). A DNA fragment containing *TRP1* gene of budding yeast (Struhl *et al.,* 1979) showed high frequency transformation and later on *ARS1* was reported adjacent to the *TRP1* gene (Stinchcomb *et al.* 1979). Similarly, *ARG4* gene (Hsiao and Carbon 1979) and a fragment coding for rRNA (rDNA, Szostak and Wu 1979) have shown high frequency transformation in yeast suggesting that these fragments replicate autonomously in yeast cells. These small genomic DNA fragments were called as ARS elements because the plasmids bearing them replicated autonomously in yeast cells (reviewed in Campbell and Newlon 1991). These plasmids transformed yeast cells with hundreds of times increased frequency as compared to the non-ARS plasmids. These results suggested that ARS elements provided the origin function in a '*cis'* acting manner and enabled the plasmids to be maintained as extra chromosomal genetic material inside the yeast cells (Kingsman *et al.,* 1979; Stinchcomb *et al.,* 1980). Moreover, the plasmids bearing them replicated once in each cell cycle (Zakian and Scott 1982; Fangman *et al.*,1983) suggesting that these elements shared regulatory controls with chromosomes. The distribution frequency of these ARS elements was estimated once per 32-40 kb (Beach *et al.,* 1980; Chan and Tye 1980, 1983). These results were very similar to those obtained from the electron microscopic studies that were used to map spacing between chromosomal replication origins or inter-origin distances (Newlon and Burke 1980).

Later on, a number of sequences conferring high frequency transformation were isolated from *S. cerevisiae*. For example, a replicator near the Glutamate tRNA gene (Feldmann *et al.,* 1981); *HO ARS* (Kearsay 1984); *ARS1* (Celniker *et al.,* 1984); *H4 ARS* (Bouton and Smith 1986); *C2G1 ARS* (ARS Palzkil *et al.,* 1986), *ARS1501* (Raychaudhuri *et al.,* 1997) and several ARS elements in a long stretch of chromosome III (ring chromosome; Newlon *et al.,* 1986, 1991), chromosome V (Ferguson and Fangman 1992), chromosome VI (Friedman *et al.* 1997), chromosome XIV (Friedman *et al.,* 1996) and chromosome XV (Raychaudhuri *et al.,* 1997) etc. Shirahige and co-workers (1993) identified nine ARS elements in a 230-kb stretch of chromosome VI covering 80% of the chromosome. Most ARSs are present as single-copy DNA in the genome with the exception of two families of repetitive ARSs. These are the ARSs that are located on the tandemly repeating rDNA units on one arm of chromosome XII and those that are associated with the telomeres of yeast chromosomes (Chan and Tye 1980; Kouorina and Larionov 1983).Origins can be classified as strong or weak, depending on the mitotic stabilities of plasmids containing them. Strong origin are the origin, which fire in almost all the cell cycles (>95%) while weak origins fire in a subset of cell cycle (<5%). Strong ARS elements promote high mitotic stabilities, and weak ARS elements promote lower mitotic stabilities.

As mentioned above, the properties of ARS elements suggested that they may serve as replication origins. However, the direct evidence that the ARS elements functioned as origins became available only in the year 1987, when two separate groups used two different techniques to demonstrate independently the physical coincidence of the 2μ plasmid replication origin with nearby ARS elements. These techniques are named as two-dimensional agarose gel electrophoresis (2D) techniques. Both the techniques separate replicating DNA molecules on the basis of their molecular weight in first dimension electrophoresis. In second dimension of neutral/neutral 2D technique of Brewer and Fangman (1987), replication intermediates (RIs) are separated on the basis of their molecular weight as well as shape,

which depend upon the presence, absence or location of the origin within the restriction fragment. Thus the 'bubble' shaped RIs formed due to the presence of internal origins are separated from the 'Y' shaped RIs formed due to the absence of internal origins causing replication of the fragments by forks coming from external origins. These fragments can be seen after southern hybridization using suitable radioactive probes (Brewer and Fangman 1987). The second dimension electrophoresis of the neutral/alkaline technique (Huberman *et al.,* 1987; Nawotka and Huberman 1988) is run under denaturing conditions so that the nascent strands of different sizes are fractionated. The restriction fragments generated from replicating DNA contain short nascent strands and the length of these short fragments at any point within the restriction fragment shows the distance of that point from its replication start point/initiation site. This technique is capable of measuring the sizes of various nascent strands generated by replicating restriction fragments and thus, can help to identify the direction of DNA replication and subsequently, the point from where the bi-directional replication starts – the origin.

Soon after the pioneering origin mapping studies in *ARS1* and yeast 2μ plasmids (Brewer and Fangman 1987; Huberman *et al.,* 1987), a chromosomal origin was located near *ARS305* of yeast chromosome III using the N/A 2D technique (Huberman *et al.,* 1988). The subsequent years witnessed a boom in chromosomal origin mapping studies in yeast and other organisms. The rDNA repeats of chromosome XII (Linskens and Huberman 1988) and the three ARS elements on the left arm of yeast chromosome III, *ARS306* (Deshpande and Nwelon 1992, Zhu *et al.,* 1992a), *ARS307* (Greenfeder and Newlon 1992) and *ARS309* (Greenfeder and Newlon 1992) are some of the examples of the earliest mapped chromosomal origins using the 2D techniques. Although the replication origins were mapped at or near the ARS elements using 2D techniques, it was not clear whether the ARS sequences were sufficient for the origin function in the chromosome or some other factors were responsible for their function. Deshpande and Newlon (1992) deleted the chromosomal copy of *ARS306* from chromosome III and replaced *ARS307* with a mutated copy of *ARS307* containing a mutation in the essential domain A. 2D gel analysis of the relevant regions showed abolition of origin activity suggesting that the chromosomal origin function was a property of ARS elements themselves.

Later on several chromosomal origins were mapped in long stretches of chromosomes using 2D gel technique in *S. cerevisiae*. In 1991, Newlon and co-workers identified a total of 14 ARS elements with an average spacing of ~14 kb from a ~ 240-kb region from right arm of chromosome III (Newlon *et al.,* 1991). Six of them, *ARS305*, *ARS306*, *ARS307*, *ARS08 ARS09* and *ARS10* were found to be active as chromosomal origins by 2D gel analysis. Poloumienko *et al.*, (2001) reported seven ARS elements from a ~140-kb region (right to MAT locus) cloned from the right arm of chromosome III. One additional ARS element, *ARS320,* was reported near *ARS303* (Vujcic *et al.,* 1999) from this region. Out of these eight ARS elements four were found active as chromosomal origins in <10% cells and two of them were active in >90% of the cells (Poloumienko *et al.,* 2001). Thus, out of total 19 ARS elements (*ARS300- ARS310* and *ARS313 – ARS320*) mapped on chromosome III twelve were identified as functional chromosomal origins (Newlon *et al.,* 1991; Poloumienko *et al.,* 2001). In another study, four origins (*ARS1411*, *ARS1412*, *ARS1413* and *ARS1414*) were identified on a 157-kb stretch of chromosome XIV that activated late in S phase (Friedman *et al.,* 1996). In 1997, two independent ARS and 2D origin mapping studies covering two largely overlapping 270-kb regions of chromosome VI reached to similar conclusions. Friedman *et al.* (1997), who identified 10 ARS elements in this region, found that three of them, *ARS603*, *ARS606* and *ARS607,* function efficiently as chromosomal origins while *ARS601/602*, *ARS605* and *ARS609* are functional in a subset of cells and *ARS608* is inefficient. Yamashita *et al.,* (1997) mapped a total of 10 ARS elements from this region and found that chromosome VI contains 3 replication origins which function efficiently in every cell cycle and two of them were active in less than 5% cells (total five chromosomal replication origins) under normal growth conditions. The initiation efficiency of replicators varies from one replicator to another and some of the replicators are not active as chromosomal replication origins (Dubey *et al.,* 1991; Newlon *et al*., 1993; Friedman *et al*., 1997; Yamashita *et al*., 1997). It was suspected that the proximity to telomere might be the reason for its inactivity. To check the possibilities, one of such ARS element *ARS301* was moved to relatively internal site at the same chromosome III. Even after changing the location of *ARS301*, it was still inactive and the results suggested that some of the ARS element do not always function as chromosomal origin. Probably, a number of other factors are there, which influences chromosomal activity of an ARS element (Dubey *et al.,* 1991; Deshpande and Newlon 1992). Similarly, when *ARS302* and *ARS303* were tested on a ring chromosome (does not contain telomere) neither *ARS302* nor *ARS303* was detectably active as chromosomal replicator, hence, shows that telomere has no effect on ARS activity. It was further shown that *ARS301* region of chromosome III was replicated by a fork moving towards the telomere from the distant *ARS305* origin (Huberman *et al.,* 1988). Later on several chromosomally inactive ARS elements were also reported.

Sequence comparison between different ARS elements identified an 11 bp ARS consensus sequence (ACS) as a specifically conserved element (A/T)TTTATPuTTT(A/T) (Stinchcomb *et al.,* 1981; Broach *et al.,* 1983). Similarly, other ARS elements have been analysed and an 11 bp core sequence with mismatches was found necessary for ARS /origin function but internal deletions analysis showed they are not sufficient for ARS function (Celniker *et al*., 1984; Kearsey 1984; Bouton and Smith 1986). Later on, the boundaries of ARS elements were defined by *in-vitro* nested deletions while protein binding sites and structural motifs were identified by linker substitution analysis. Genetic and mutational analysis revealed that *S. cerevisiae* ARS elements contain three essential functional domains namely domain A, B and C. The domain A contains a conserved sequence of 11 bp, the ACS, which is essential for origin activity since it contains the recognition site for initiator proteins (ORC). Domain A is flanked by A+T rich domain B at the 3′ end of T rich strand of ACS while domain C, present at 5′ end of the core consensus sequence, has less sequence specificity as it varies in different ARS elements (reviewed in Campbell and Newlon 1991; Marahrens and Stillman 1992). Domain B is further subdivided into three sub domains named B1, B2, & B3. Although the 3′-flanking regions of all ARS elements are A/T-rich, they display little sequence homology and do not contain common consensus sequence elements. Progressive external deletions that enter domain B gradually reduce and eventually abolish ARS activity. Domain B1 is required for efficient binding of ORC proteins (Rao and Stillman 1995; Rowley *et al.,* 1995). Domain B3, present in a subset of ARS elements, contains a consensus sequence acting as binding site for the yeast DNA-binding protein Abf1, which stimulates ARS activity (Marahrens and Stillman 1992).

In addition to the ACS (domain A), *ARS1* (Marahrens and Stillman 1992) and *ARS307* (Rao *et al.,* 1994; Theis and Newlon 1994) contain B1 and B2 elements 3′ to the T-rich strand of the ACS. The B1 sequences at *ARS1* and *ARS307* include sequences important for ORC binding (Rao and Stillman 1995; Rowley *et al.,* 1995), and it is now recognized that ORC contacts a bipartite DNA sequence consisting of nucleotides in the ACS and B1 elements (Lee and Bell 1997). The *ARS1* B3 element contains an Abf1p binding site. *ARS302* (Sharman *et al*. 2001), *ARS305* (Crampton *et al*., 2008; Lin and Kowalski 1997), and *ARS315* (Crampton *et al*., 2008) contain B1 and B2 elements as well, and studies of several ARS elements on chromosome VI have shown that domain B is important for origin activity (Rashid *et al.,* 1994). Some ARS elements also contain stimulatory sequences 5′ to the T-rich strand of the ACS in domain C. Stimulating sequences seems to consist essentially of two elements: a sequence specific element, which thought to be a protein binding sequence and a non-sequence element that is thought to function as a DNA unwinding element (DUE) (Deshpande and Newlon 1992;

River and Rine 1992; Shirahige *et al.,* 1993; Marahrens and Stillman 1994; Rashid *et al.,* 1994, Theis and Newlon 1994; Huang and Kowalski 1996). For several of these ARS elements, domain C contains transcription factor binding sites for Abf1p (Walker *et al.,* 1990), Rap1p (Sharma *et al.,* 2001), Mcm1p (Chang *et al*., 2004), or Sum1p (Irlbacher *et al.* 2005) that stimulate, but are not required for, origin activity. Fine analysis of *H4 ARS* and *ARS121* have shown that in addition to the ACS, a 66 bp of 3′ flanking domain in *H4 ARS* and T-rich strand (ATR ~72 bp) located at 3′ end along with two OBF1 DNA-binding sites and located at 5′ end in *ARS121* were found necessary for the ARS function (Holmes and Smith 1989; Walker *et al*.,1990). The OBF1 DNA-binding site was proved as an enhancer of DNA replication and suggested that the DNA-binding site and the OBF1 protein are involved in the regulation of the activation of nuclear origins of replication in *S. cerevisiae* (Walker *et al*., 1990). The ARS consensus sequence (ACS) shows conserved nature in most of the ARS elements hence it was recognized as a site for the binding of necessary proteins that help in the initiation function. Later ORC, a six-protein complex, was identified, which binds to ACS in ATP dependent manner and helps in initiation process (Bell and Stillman 1992; Diffley and Cocker 1992). The ORC provides binding site for other additional proteins that help in the regulation of replication initiation (reviewed in Bell 2002). Mutational analysis of ORC genes has shown the loss of origin function and prevention of binding of other proteins at the ACS (Bell *et al.,* 1993; Bell 2002).

The homologous conserved proteins similar to yeast ORC were also identified in other organism like *Xenopus*, *Drosophila* and Human (Bell 2002) suggesting that the replication processes in various eukaryote follow some common mechanism. Further analysis show that binding of ORC to ACS is facilitated by domain B1 and domain B2 that provides DNA unwinding sites having low helical energies (Natale *et al.,* 1993). Recently, it has been shown that domain B2 is required for the assembly of pre-replication complex (Wilmes and Bell, 2002) and domain B3 acts as enhancer by binding transcription factor Abf1 suggesting a close relationship between replication and transcription. Soon after the completion of whole chromosome studies, attempts were made to study at whole genome level using advance technologies. Brewer and Fangman's group, the first genome wide analysis of origins, used advanced DNA microarray technology to determine the time of replication of thousands of sites across the genome by hybridizing replicated and un- replicated DNAs isolated at different time points in mid S phase when most of the origins fire and identified a total of 332 origins (Raghuraman, *et al.,* 2001). DNA ChIP (chromatin immuno-precipitation) technology was used for the identification of origin recognition complex (ORC) and mini chromosome maintenance (MCM) proteins from pre-replicative complexes at origins of replications and they have located a total number of 429 potential origin sites (Wyrick *et al.,* 2001).

Another approach utilising microarray analysis of copy number changes from one to two during DNA replication in synchronised (G1 blocking by α factor) yeast cells identified a total of 260 potential replication origins (PROs; 143 were early replicating while 104 were late replicating, and 13 origins at chromosomal terminus) in the entire yeast genome, which fire in the presence of hydroxyurea (Yabuki *et al.,* 2002). Single stranded DNA (ssDNA), that forms during the replication process, analysis in wild type (WT) and checkpoint deficient rad53 yeast cells challenged with hydroxyurea (HU) identified 113 ssDNA locations in WT cells and 315 clustered ssDNA peaks (106 Rad53-unchecked origins and 209 Rad53-checked origins) from *rad53* cells. Ninety-six of the 106 Rad53-unchecked origins co-localize to regions previously identified as origins that increase in copy number in WT cells in HU15 (Feng *et al.,* 2006). Tiled oligonucleotide array (NimbleGen) approach was used to map the ORC and MCM2 protein binding sites at high resolution, which define a set of potential Autonomously Replicating Sequences (ARSs) binding site, called as nimARSs and 529 nimARS sites were identified including 349 ORC-MCM2 sites, 178 MCM2 only sites and two ORC only sites, which include 95% of known ARSs, and experimental verification demonstrates that 94% are functional (Xu *et al.,* 2006). The results of above studies were cross verified by localizing the previously reported ARS/origins and shown considerable overlapping. The genome wide locations of origins were also identified by computational method. An oriscan algorithm was developed using similarity to 26 previously characterized origins identified 268 bp of sequence, including the T-rich ACS and a 3′ A-rich region, which identified a lot of location of the ACSs in the genome, which correspond to ORC binding site (Breier *et al.,* 2004). Similarly, phylogenetic conservation analysis in *S. cerevisiae* identified replication origin sequences throughout the genome at base pair resolution and origin activity was confirmed for each of 228 predicted sites representing 86% of apparent origin regions (Nieduszynski *et al.,* 2006). Thus, starting from the localization of the first chromosomal origin in 1988, the realization of genome wide origin mapping by 2011, marked a great progress in the area and paved the way for similar studies in other organisms.

Replication studies in fission yeast

The fission yeast, *S. pombe* is a haploid yeast that shares many similarities with higher eukaryotic cells, i.e., sub-cellular organelles, cytoskeleton and nucleosome organization of chromosomal DNA. Its chromosomes condense during mitosis while those of *S. cerevisiae* do not. Its mode of cell division, presence of a distinct G2 phase and visible condensation of chromosomes during mitosis are very similar to higher organisms. Because of its small genome size, ease of genetic manipulation and availability of a reliable ARS assay (Maundrell *et al.,* 1988), *S. pombe* is a suitable model for the study of ARS elements and origins to answer DNA replication related questions in higher eukaryotes and animal cells. The ARS assay and 2D techniques have been extensively used for replication origin studies in *S. pombe* (Maundrell *et al.,* 1988; Caddle and Calos 1994; Dubey *et al.,* 1994; Okuno *et al.* 1997; Srivastava and Dubey 2007; Dubey *et al.,* 2010). Of the limited number of ARS elements mapped in *S. pombe,* only a few have been analysed in detail (reviewed in Masukata *et al.* 2004). The fission yeast ARS elements are several-fold larger than those of *S. cerevisiae* ranging between 500-1500 bp. They are extremely AT rich, do not contain any essential common consensus sequence like ACS of *S. cerevisiae* replication origins (Maundrell *et al.* 1988) but have asymmetrical As and Ts, i.e., multiple As on one strand and multiple Ts on the other strand, which are required for the ARS activity (Zhu *et al.* 1994; Clyne and Kelly 1995; Dubey *et al.* 1996; Kim and Huberman 1998; Okuno *et al.* 1999; Segurado *et al.* 2003; Dai *et al.* 2005).

Extensive origin mapping studies have revealed the presence of a large number of potential origins in *S. pombe* chromosomes most of which are inefficient, firing only in a small fraction of a cell population (reviewed in Masukata *et al.* 2004). Many of these origins do not function as ARS elements when cloned in a plasmid as a monomer suggesting that, in *S. pombe*, the relationship between ARS and origin activity is different from *S. cerevisiae* (Dai *et al.,* 2005; Dubey *et al.,* 2010). They are clustered more often than the *S. cerevisiae* origins and some of them seem to contain sequences influencing their efficiency and firing time. Association of an enhancer element with *ars3002*, the efficient ARS of the *ura4* origin region on chromosome 3, has been reported (Kim and Huberman 1999). In 2004, Yompakdee and Huberman reported a sequence element associated with *ars727* on chromosome 2 that appears to control the timing of initiation of the nearby ARS elements in plasmids. Interestingly, *ars727* has been found to be inactive as a chromosomal origin (Kim and Huberman 2001) and there appeared to be a shifting of replication timing from early to late within 10-kb of *ars727* in chromosome 2 (Dubey *et al.,* 2010). These observations strongly suggested the possibility that the plasmid replication time determining elements associated with *ars727* play a similar role in chromosomes too. The yeast transformation experiments done during eighties provided the first indication that DNA replication in *S. pombe* could be sequence specific. Initially, taking the advantage of

yeast transformation technique, Autonomously Replicating Sequence (ARS) element was reported in the vicinity of *ura1* gene (Sakaguchi and Yamamoto 1982), which was capable of replicating autonomously suggested the existence of ARS elements in *S. pombe*. Later, a number of autonomously replicating sequences were reported in *S. pombe* i.e. two DNA fragments (1.1-kb *ars1* and 1.3-kb *stb* fragment) cloned along with *URA3* gene of *S. cerevisiae* (Heyer *et al.,* 1986; Losson and Lacroute 1983), Ribosomal RNA (rRNA) genes present on chromosome III (Toda *et al.,* 1984), *ars2PL* and *ars2PR* at the silent P mating type locus located near promoter region of *mat2* locus (Olsson *et al.,* 1993) etc. To explore more about the nature and function of ARS elements, 78 fragments were randomly cloned in MAK262 vector, an ARS deficient shuttle vector containing *LEU2* gene of *S. cerevisiae* that complements *leu 1-32* mutation in *S. pombe*, covering ~230-kb (1%) of *S. pombe* genome. Out of these 78 cloned fragments, 12 were identified as ARS elements (pSp14, 18, 23, 45, 59, 108, 112, 133, 70, 38, 107 and 48) on the basis of their transformation frequencies with an average ARS frequency of 19-kb or twice the frequency in *S. cerevisiae*. The study also suggested that most plasmids maintain themselves as monomer or simple head to tail multimers of original plasmid and in un-rearranged form within the yeast cells but a few may be rearranged due to recombination (Maundrell *et al.,* 1985).The plasmids containing ARS elements produce genetically unstable transformants similar to *S. cerevisiae* and maintain themselves in extra chromosomal state. Thus, the true ARS elements are maintained extra-chromosomally and un-rearranged plasmids, which could be identified by southern blotting (Wright *et al.,* 1986).

Johnston and Baker 1987 for the first time attempted to characterize the ARS elements by external deletions in *ars17*, a 1.8-kb *Hin*dIII/*Eco*RI AT rich (85 %) fragment, located between two divergently transcribed genes TSL17 and CDC17 in *S. pombe*. External deletion analysis revealed a progressive loss of transformation ability with increasing size of the deletion. For instance, when a deletion of 320 bp was introduced at one end of the 1.8-kb fragment, there was a decrease from 7,466 to 4,655 transformants/µg DNA. Further deletion resulted in severe loss of activity but major loss was seen when 220 bp core sequence was deleted. Thus, the study suggested that various segments of ARS element had varying effect on ARS function and core region has severe effect probably due to presence of essential sequence for ARS activity (Johnston and Baker 1987). To better understand the ARS elements of *S. pombe*, sequence analysis was performed for the first time by Maundrell *et al.,* (1988). In this study, they cloned *Sau*3A digested *S. pombe* genomic DNA in a self-made shuttle vector MPL19, which contains *LEU2* gene of *S. cerevisiae* complementing *leu 1-32* mutation in *S. pombe*. Total eight un-rearranged ARS elements (p*ars*727, p*ars*744, p*ars*745, p*ars*747, p*ars*756, p*ars*766, p*ars*767 and p*ars*772) were identified during the study by high frequency transformation in *S. pombe*.

Their sizes varied between 0.8-1.8-kb and the AT content was found relatively high (69-75%) compared to the *S. pombe* genome as whole (55%). A 1.2-kb *Eco*RI fragment (pFL201.2), isolated from pFL20 (Losson and Lacroute 1983), was also sequenced for the comparison during the study. Sequence comparison studies revealed a 11 bp ARS consensus sequence (ACS) 5′(A/T)PuTTTATTTA(A/t) 3′ in all the ARS elements similar to *S. cerevisiae* consensus sequence 5′ (A/T)TTTATPuTTT(A/T) 3′ (Broach *et al.,* 1983). Deletion mapping had shown the presence of consensus sequence within the vicinity of functional ARS element, however precise excision of 11 bp consensus sequences by *in vitro* mutagenesis has little effect on the ARS activity in *S. pombe*, contrarily consensus sequences were absolutely required for ARS activity in *S. cerevisiae*. Since consensus sequences were found to be present in all the ARS elements, it was argued that they probably play some important role *in situ* in the chromosome probably were required for the DNA – protein interaction for the proper ARS function (Maundrell *et al.,* 1988).

Following the discovery of ARS elements as the putative replicators in *S. cerevisiae*, different groups started looking for similar elements in *S. pombe*, which had became an attractive laboratory model organism because of its several resemblance with higher organisms including its chromosome condensation during mitosis which is absent in *S. cerevisiae* (Allshire *et al.,* 1987). It shows autonomous replication activities and a reliable ARS assay was available for *S. pombe* (Maundrell *et al.,* 1988). However, it was not clear whether these ARS elements correspond to chromosomal origins or not. It was thought that origins in this organism will resemble to higher eukaryotes and hence can be used as a good model for the study of higher eukaryotic or animal cell origins (Zhu *et al.,* 1992b). To better understand the nature of origins in *S. pombe*, a 16.9-kb fragment of chromosome III including *ura4* gene was tested by neutral/neutral (N/N) and neutral/alkaline (N/A) 2D gel electrophoresis technique and led to the identification of an origin located 4-8-kb upstream of the *ura4* gene with a initiation zone of >4-kb where the fork moved only out of this region without any trace of inward movement. Since the *ura4* origin was the first origin mapped in *S. pombe* but unfortunately, it could not be mapped precisely by 2D gel technique, it was considered as a complex origin (Zhu *et al.,* 1992b) similar to the previously mapped animal cell origins– DHFR origin in CHO cells and *Drsophila* chorion gene origins.

Further analysis of the *ura4* origin region by 2D gel technique showed multiple initiation sites within it. Total three ARS elements (*ars3003* at left hand and *ars3002*, *ars3004* at right hand side) were mapped in this region. The deletion of *ars3002* or *ars3003* resulted in either dramatically reduced or completely diminished initiation activity from these regions and the ARs remaining in the immediate vicinity of deletion became stronger. The lack of termination signals within the *ura4* region suggested that one of the three origins fires at a time (Dubey *et al.,* 1994). 2D gel analysis of a 6.2-kb fragment and its 2.8-kb sub clone suggested that the replication initiates in a specific region localized within several hundred base pairs in both the fragments, which also function in its chromosomal native location. Thus, it seems that chromosomal origins co-localize with ARS elements and initiation events require specific sequence or elements in *S. pombe* (Caddle and Calos 1994). In another study, total 47 fragments were cloned from *S. pombe* representing 241-kb DNA. Four of them showed 5640 large colonies/µg DNA while another four showed intermediate transformation frequency of ~ 800 colonies/µg DNA and the remaining 39 clone showed small colonies and a low transformation frequency. Thus, a total five ARS elements (*ars*1-1, *ars*2-1 , *ars*2-2, *ars*2-3 and *ars*3-5) were identified in a region covering almost 277 kb of *S. pombe* genome (Wohlgemuth *et al.,* 1994; Caddle and Calos 1994). This resulted into an average frequency of one origin per 55-kb. 2D gel analysis revealed that three of them have initiation events in specific location while in the fourth fragment (contains two discrete origins) initiation occurs within a 3-kb zone. It was also suggested that all of them were used as chromosomal origins but only in a subset of cells or cell divisions (Wohlgemuth *et al.,* 1994).

Two extensive ARS and 2D origin mapping studies were done on a 100-kb stretch (Okuno *et al.,* 1997) and a different 75-kb stretch (Srivastava and Dubey 2007; Dubey *et al.,* 2010) of *S. pombe* chromosome II. Both of these regions were systematically cloned such that the overlapping clones covered almost all the region of interest. These clones were tested for the ARS activity followed by 2D gel analysis. The first study revealed the presence of five ARS elements (*ars2001*, *ars2002*, *ars2003*, *ars2004* and *ars2005*) with in the 100-kb stretch. Out of these five ARS elements three (*ars2002*, *ars2003* and *ars2004*) were chromosomally active while two other (*ars2001* and *ars2005*) were inactive in their native location but active on plasmid. The *ars2004* or *ori2004* produced a strong bubble signal similar to a strong *S. cerevisiae* origin suggesting that it was very efficient, active in almost all the cell cycles while other two *ars2002* (*ori2002*) and *ars2003* (*ori2003*) were less efficient, active once every few cell cycles (Okuno *et al.,* 1997).

Similarly, the second analysis revealed the presence of six ARS elements (five new ARS elements and one previously mapped *ars727*, Maundrell *et al.,* 1988). These were distributed in two clusters in such a way that *ars2006*, *ars2007* and *ars2008* were close to each other making one cluster while *ars2009* and *ars2010* formed another cluster (Srivastava and Dubey 2007). Thus, the clustering of these ARS elements appeared similar to the ARS cluster of the *ura4* origin region (Dubey *et al.,* 1994), *ars*1-1 cluster (Wohlgemuth *et al.* 1994) and ARS clusters found in the centromeric regions (Smith *et al.,* 1995, reviewed in Masukata *et al.* 2004) in *S. pombe*. Analysis by the 2D technique revealed the presence of total 5 origins in the 75-kb region – three origins, *ori1*, *ori3* and *ori5* were moderately active and the other two (*ori2* and *ori4*) were very weak. DNA fragments containing the three moderately active origins and one weak origin, *ori2*, were ARS positive whereas the one containing the other weak origin, *ori4*, was ARS-negative. Three ARS elements reported earlier from this region appeared to be inactive as chromosomal origins (Dubey *et al.,* 2010).

An autonomously replicating sequence (ARS) element, *ars3001*, has been mapped to a 2.3-kb restriction fragment of 10.9-kb rDNA repeats (100-150 copy of gene) on chromosome III spanning the junction between the non-transcribed spacer (NTS) and the external transcribed spacer upstream of the ribosomal RNA genes. The 2D gel analysis of the fragments has shown that replication initiation occur in the NTS portion of the same fragment. A pause region at the 3′ end of the rRNA genes inhibits forks from entering these genes counter to the direction of transcription (Sanchez *et al.,* 1998). Thus, the above results suggest that *S. pombe* cells contain both discrete as well as clustered origins however the proportion is not known. The origins may be efficient or inefficient based on their firing in every cell cycle or in few cell cycles, respectively (Okuno *et al.,* 1997). A subset of these origins was utilized during S phase of cell cycle. Probably other origins are inactivated by the passage of replication fork of nearby origins that fire little early in the S phase of cell cycle (Masukata *et al.*,2004).

The average frequency of ARS elements was estimated one per 20-50-kb (Maundrell *et al.,* 1985; Caddle and Calos 1994; Wohlgemuth *et al.,* 1994). Fission yeast ARS elements co-localize with chromosomal origins of DNA replication and, when tested, were shown to be required for initiation of DNA synthesis at these sites (Caddle and Calos 1994; Dubey *et al.,* 1994; Wohlgemuth *et al.* 1994). Sequence analysis of a number of *S. pombe* ARS elements revealed the presence of an 11 bp consensus known as the *S. pombe* ARS consensus sequence, which is different from the ARS consensus sequence (ACS) identified in *S. cerevisiae* (Maundrell *et al.,* 1988). Genetic analysis indicates, however, that the *S. pombe* ACS were not essential for plasmid ARS activity in *S. pombe* (Maundrell *et al.,* 1988; Zhu *et al.,* 1994).

The average length of fragments capable of sustaining autonomous replicating sequence (ARS) activity was shown to be 500–1500 bp (Dubey *et al.,* 1994; Zhu *et al.,* 1994). To understand the nature of ARS elements, deletion and linker substitution analysis was performed for a number of ARS elements (Clyne and Kelly 1995; Dubey *et al.,* 1996; Kim and Huberman 1998; Okuno *et al.,* 1999). All the mutational analysis in plasmids revealed lack of a consensus sequence, which could define an origin and shown that the essential sequences for replication initiation were not same. First fine-structure genetic analysis was reported for *ars1*, an A/T-rich (74% A+T content) 1.2-kb sequence (Heyer *et al.,* 1986) present in most *S. pombe* replicating vectors. Systematic mutagenesis of *ars1* showed that the minimal segment required for ARS activity was 800 bp, quite large in comparison to *S. cerevisiae* ARS elements. 50 bp internal deletion analysis of *ars1* demonstrated the existence of two important segments, segment 1 at one end that is essential for ARS activity and segment 9 on the other end reduced ARS activity as much as 5-fold. Most of the remaining fourteen 50 bp deletions had little effect on ARS activity. All mutations that removed - substantial portions of segment 1 greatly reduced ARS function. However, 10 bp linker substitutions within the segment 1 had little effect on ARS activity, indicating that there may be some functional redundancy of the genetic elements required for full *ars1* function. Further analysis of 30 bp A/T rich segment indicated that the right-most 20-30 bp contains sequences that make significant contributions to ARS function. This core sequence has a marked asymmetry of A and T residues with most of the Ts on one strand and most of the As on the other. Sequences similar to this 30 bp core sequence element are also found in functionally important regions of other *S. pombe* ARS elements for example *ars17* (Clyne and Kelly 1995). Similar studies were performed for fine analysis of *ars3002* spanning 821 bp. It showed the presence of two essential modules (8 & 10) of 30-55 bp and at least two other important modules in *ars3002* as compared to *ars1*, which contained one important and one additional important module. The two essential regions of *ars3002* were similar to each other and similar sequences were also found in other ARS elements suggesting that either one of them or both might function as ACS in *S. pombe* similar to *S. cerevisiae* (Dubey *et al.,* 1994).

Fine analysis of *ars3001* (Kim and Huberman 1998) and *ars2004* (Okuno *et al.,* 1999) produced similar results through deletion and linker substitution experiments. The studies of *ars3001* (~570 bp) revealed that it contained multiple important regions three of which, namely "α, β and γ", were very important as they were essential for origin function but only when they were placed in correct orientation. Each of these regions were asymmetrically A + T rich containing sequence similar to ACS and they could functionally replace each other suggesting a synergistic interaction for binding initiation proteins for ARS as well as origin function (Kim and Huberman 1998). Internal deletion analysis of *ars2004*, an intrinsic chromosomal replication origin (a 940-bp fragment, *ars2004M*) showed three important regions I, II and III, required for autonomously replicating sequence (ARS) activity. Eight base pair substitutions in the 40-bp region I composed of arrays of adenines on a DNA strand resulted in a great reduction of ARS activity. Substitutions of region I with synthetic sequences showed that repeats of three or more consecutive adenines or thymines without interruption by guanine or cytosine were required for the ARS activity. The 65-bp region III contained 11 repeats of the AAAAT sequence, while the 165-bp region II had short adenine or thymine stretches and a guanine- and cytosine-rich region which enhanced ARS activity. All three regions in *ars2004* could be replaced with 40-bp poly dA/dT fragments without reduction in ARS activity. Although spacer regions in the *ars2004M* enhanced ARS activity, all of them could be deleted when a 40-bp poly dA/dT fragment was added in place of region I. It seems that the origin activity of fission yeast replicators depends on the number of adenine/thymine stretches, the extent of their clustering, and presence of certain replication-enhancing elements (Okuno *et al.,* 1999).

The above-mentioned deletion and substitution studies suggested that *S. pombe* ARS elements are composed of multiple redundant elements, the sequential excision of which did not have an all-or-none effect, but led to a gradual decrease in origin function. It appears that there are more than one sequence motifs with internal redundancy important for their function. For example, deletion of rightmost any one of the three 50-bp segments of *ars1* reduces transformation frequency more than 2-fold but deletion of all the three 50 bp segments together reduces transformation frequency 50-fold (Clyne and Kelly 1995). Thus, the important sequences within the rightmost 150 bp of *ars1* are redundant for ARS function and only by deleting enough of them shows their importance. Segments whose individual deletion seriously impairs ARS activity probably play unique role in ARS function and the segments whose deletions do not seriously inhibit ARS activity may also make important contributions. Both *ars3001* and *ars3002* contain multiple segments whose deletion seriously impaired ARS activity, and *ars3002* contain two non-contiguous segments (Dubey *et al.,* 1996; Kim and Huberman 1998) whose deletion eliminated ARS activity.

Two-dimensional gel electrophoresis of *S. pombe* origins suggested that DNA replication starts at discrete genomic loci that can be mapped to few base pairs (Dubey *et al.,* 1994, Gomez and Antequera 1999; Zhu *et al.,* 1992b). Homology search studies revealed that origins (ORIs) were the places within the promoter or intergenic regions upstream of genes (Gomez and Anequera, 1999). For example, *ura4* region shows the presence of three closely spaced origins localized within the promoter regions of three adjacent genes (Dubey *et al.,* 1994). Similarly, mapping of genomic *ars1* replication origin at nucleotide resolution have shown that replication initiates at a defined position immediately upstream from the *hus5* gene promoter (Gomez and Antequera 1999). Soon after the completion of *S. pombe* genome sequencing project in the year 2002, the genome-wide analysis of the nature and function of *S. pombe* origins became feasible.

A genome wide analysis was performed on the basis of A+T rich islands by the help of bioinformatics tools to find out the AT rich islands (AT content >72% in a 0.5–1.0-kb window). AT-rich islands were shown to reside in intergenic regions and to be over-represented between divergent transcriptional units. The study revealed the presence of 384 such A + T rich regions, which predicted the location of most of the origins within the genome. Average origin density was estimated to be one every 33-kb, with a higher density in centromeric, subtelomeric and mating-type loci (about four-fold higher than the genomic average). Analysis of 20 randomly chosen A+T rich islands by 2D gel technique revealed that ~90% of them (18 islands) function as efficient replication origins in their native locations suggesting that, out of 384 A+T islands, 345 may function as origins in the genome (Segurado *et al.,* 2003). These A + T rich sequences are probably important for the binding of *S. pombe* ORC (SpORC). The SpORC4 protein specifically binds to AT hook domain (Lee *et al.,* 2001, Kong and DePamphilis 2001). It was shown that the properties of known fission yeast origins were similar to fission yeast intergenic regions in general (Dai *et al.,* 2005). Out of 26 intergenic regions tested on plasmids for origin function, four functioned as monomers and 10 functioned as dimers, leading to the suggestion that approximately half of fission yeast 4978 intergenic regions may have potential origin function. A lack of a consensus sequence was also noted, while AT content and intergenic sizes were suggested to constitute the major determinants of origin activity. Based on these findings, a hypothesis was formulated, suggesting that DNA replication is stochastic in nature and out of many potential origins across the fission yeast genome, only a few will fire in any given cell cycle (Dai *et al.,* 2005).

Further evidence for the stochastic nature of origin selection was given by Patel *et al.* 2006. Labelling of newly synthesized DNA with thymidine analogues in the presence of hydroxyurea (to arrest fork progression), as well as pulse labelling of DNA in early and late S-phase, was used to identify replication intermediates. These intermediates were analysed at the single-cell level with DNA combing. In combination, fluorescence *in situ* hybridisation (FISH) was used to map the replicating areas on specific genomic locations. 1000 inter bubble distances were measured and the distribution of inter-origin gaps was found to be exponential, which clearly indicates random initiation pattern across the genome. The stochasticity of origin firing was further supported by the analysis of origins at the *ura4* and *nmt1* regions. The firing probabilities of origins in these regions were in the approximate range 10–70%. In addition, this analysis showed no evidence for coordinated regulation of neighbouring origins, while labelling of DNA intermediates in two sequential S-phases showed that origin selection is random between successive cell cycles (Patel *et al.,* 2006; reviewed in Legouras *et al.,* 2006). A recent genome-wide analysis identified 321 origins in fission yeast at a resolution of 12-kb by mapping single stranded DNA on ORF microarrays in the presence of hydroxyurea (HU) in an S-phase checkpoint deficient strain (Feng *et al.,* 2006). However, only a subset of these (61%) could be identified in cells undergoing a normal S-phase suggesting that an S-phase checkpoint might suppress many origins. In a recent genome-wide study using both intergenic and ORF microarrays, 401 strong and 503 putative weaker origins were identified during the mitotic and premeiotic S-phases, resulting in an average inter-origin distance of 14-kb. It was also shown that the Rad3 dependent S-phase checkpoint does not affect the firing of origins (Heichinger *et al.,* 2006). To map origins, the increase in DNA content was monitored using the temperature-sensitive cdc25-22 mutant strain that blocks cells in late G2 (Fantes 1979) and two types of experiments were performed. In the 'time-course experiment', DNA samples were taken every 5–10 minutes until S-phase was complete. In the 'HU experiment', HU was added to slow down replication fork progression and confine DNA synthesis to the vicinity of origins The HU experiment allowed origins to be mapped more precisely because fork migration away from the origin was reduced (Patel *et al.,* 2006). Peaks from both approaches generally coincided, and by combining data from both types of experiments a total of 401 strong origins were identified. Using this method, even closely spaced origins such as *ars3003* and *ars3002/4* (3.5-kb interorigin distance) within the well-characterized *ura4* origin cluster could be identified (Dubey *et al.,* 1994; Segurado *et al.,* 2003; Patel *et al.,* 2006). Comparing the data with Segurado *et al.* 2003 and Feng *et al.* 2006, they identified that 87% and 84% origins co-localized with the 401 reported origins, respectively. The timing of replication of different chromosomal regions was also measured. Overall, Chromosome III replicated 3 minute earlier than Chromosomes I and II, and the left arm of Chromosome II replicated 2 minute earlier than right arm (Heichinger *et al.,* 2006).

Hayashi and co-workers used a high-resolution whole-genome mapping of Orc1and Mcm6 binding sites, which allowed us to identify precise locations of 460 pre-RC sites on *S. pombe* chromosomes and found that 307 pre-RC sites acted as early origins that initiated DNA synthesis in the presence of HU, whereas the rest 153 of pre-RC sites were considered as late and/or inefficient origins (called collectively as late origins). Because more than 80% of Orc1-binding sites were co-localized with Mcm6, the majority of ORC binding sites serve for pre-RC assembly. The pre-RCs are formed exclusively in long and AT-rich intergenic regions as described previously (Gomez and Antequera 1999; Dai *et al.,* 2005). Early and late origins tend to distribute separately in large chromosome regions. Interestingly, pericentromeric heterochromatin and the silent mating-type locus replicated in the presence of HU, whereas the inner centromere or subtelomeric heterochromatin did not. Notably, MCM did not bind to inner centromeres where origin recognition complex was located. Thus, replication is differentially regulated in different chromosome domains (Hayashi *et al.,* 2007).

One more genome wide study using ORF specific DNA microarray analysis had shown that the *S. pombe* origins fire with varying efficiencies and at different times during S phase. A novel approach "the near sigmoid fitting" shows that the replication efficiencies differ from origin to origin depending upon the efficiency of origin firing. The replication in early S phase seems to be inefficient due to inefficient firing of the origins while efficient replication occurs in late S phase attributed to efficient but late firing origins (Eshaghi *et al.,* 2007). Since the efficiency of firing and time of replication are two important attributes that contribute to the overall regulation of replication, a number of studies focussed on these points have been conducted in *S. cerevisiae, S. pombe* and other organisms. It has been shown that a number of ARS elements show enhancers of origin function in *S. cerevisiae.* These enhancer elements bind Abf1 protein in *ARS120*, *ARS121*, *ARS1*, *HMR-E ARS* (Shore *et al.,* 1987; Eisenberg *et al*., 1988; Sweder *et al*., 1988; Marahrens and Stillman 1992; Buchman *et al*., 1988; Walker *et al.,* 1990). Fine analysis of *ARS1501* shows that it functions as a chromosomal replication origin in chromosome XV and a replication enhancer REN1501 was reported at 5′ end to the T rich strand of the ACS that binds certain proteins necessary for the replication initiation. Recently, an ARS inhibitory element that likely influences local chromatin structure was discovered towards the 3′ end of the B2 element in *ARS305* and

ARS315 (Crampton *et al*., 2008). Reynolds and coworkers have examined 200 kilobase region containing ~60 % of the chromosome III extending from telomere on the left arm to the mating type locus (MAT) on the right arm and determined the temporal pattern of DNA replication of eight already identified ARS elements (Newlon *et al.,* 1986; Greenfeder and Newlon 1992). Timing of replication was studied by using synchronous cultures and transfers from dense to light isotope medium. The temporal pattern of mitotic DNA replication of eight fragments that contained ARSs was determined. ARS elements near the telomeres replicated late in S phase, while internal ARS elements replicated in the first half of S phase. The results suggest that some ARS elements in the chromosome may be inactive as replication origins. The actively expressed mating type (MAT) locus replicated early in S phase, while the silent cassettes, HML and HMR, replicated late in S phase. Unexpectedly, chromosome III sequences were found to replicate late in Gl at the arrest induced by the temperature-sensitive cdc7 allele. Timing experiments suggest that origins located near the centre of chromosome III are activated quite early in S phase, and the telomere of the chromosome is late replicating due to passive replication by slow moving forks initiated at distal, early origins (Reynolds *et al.,* 1989).

Although replication of circular ARS plasmids faithfully mimics that of linear yeast chromosomes with respect to single activation event per origin in a given S phase (Fangman *et al.,* 1983), the timing of origin activation can differ substantially in a plasmid and in a chromosome (Ferguson and Fangman 1992). Four replication origins (*ARS1411*, *ARS1412*, *ARS1413* and *ARS1414*) located close to telomere on chromosome XIV initiate replication at mid- to late S phase. The late activation time of at least two of these origins is maintained when the origins and flanking sequences are located on circular plasmids, indicating that the late activation of these origins is independent of the telomere. Furthermore, multiple sequences contained in the DNA flanking two of these origins influence replication initiation time (Freidman *et al*., 1996). These late determinants are functionally equivalent, because duplication of one set of contributing sequences can compensate for the removal of another set. Furthermore, insertion of an origin that is normally early activated into this domain results in a shift to late activation, suggesting that the chromosome XIV origins are not unique in their ability to respond to the late determinants (Freidman *et al.,* 1996). *ARS501* located near by the telomere on chromosome V, which replicates late in S phase, was cloned on a circular plasmid, it replicated early in S phase (Ferguson *et al.,* 1991; Ferguson and Fangman 1992) suggesting that the late replication properties are governed by the proximity to the telomere. In yeast, it appears from several studies that early replication of an origin is its default state but late replication is imposed by the neighbouring sequences (Friedman *et al.,* 1997). The temporal pattern of chromosome VI replication origins was found similar to the chromosome III – the telomeres replicated late in S phase while the central region replicated in early S phase. (Freidman *et al.,* 1997)

Later on it was shown that dormant replication origins on the left arm of budding yeast chromosome III become activated when passive replication through them is prevented by inactivating the Mec1/Rad53 checkpoint that blocks late-origin firing. Under these conditions, dormant origins fire very late relative to other active origins. These experiments show that some dormant replication origins are competent to fire during S phase and that passage of a replication fork through such origins can inactivate them (Santocanale *et al.,* 1999). *ARS302* and *ARS303* (Chromosomally inactive origins) are programmed to fire in late S phase but they are passively replicated by the fork of neighbouring early replicating origin *ARS305* before they have chance to fire. Analysis shows that both flanking and internal sequence contribute to chromosomal inactivity by programming them to fire in late S phase. In the *ARS301*, the function of internal sequences determining chromosomal inactivity depends on the checkpoint proteins Mec1p and Rad53p (Sharma *et al.,* 2001). Two earlier studies suggest that *S. pombe* ARS elements contain specific sequence elements which control their efficiency and replication timing. An enhancer (~400 bp) sequence was reported near the *ura4* gene region that stimulates the activity of nearby ARS and this function was independent of position and orientation of the enhancer element. Deletion studies revealed that 50 bp stretches within the enhancer contribute to its function in a redundant fashion. Most probably this sequence along with the sequences of core origin plays an important role in the binding of ORC proteins (Kim and Hubeman 1999). Similarly, a 200 bp Late Replication enforcing (LRE) sequence was reported, which was sufficient to compel nearby plasmid origins to fire late and they also function in orientation and position independent manner. The LRE contains three 10 bp late consensus sequences (LCS), which cooperate each other and with other sequences in 200 bp fragment to enforce late replication (Yompadkee and Huberman 2004).

Conclusion

Till now, a great deal of information has been explored in yeasts and need to be explored furthermore. Genome wide studies have given an idea of huge number of possible replication origins but actually they work or not *in-vivo*, is not yet very clear. Similarly, the number of origins identified from various techniques has a great variability in the number, which creates a doubt regarding the applicability of the experimental data. If the experimental data is correct than the actual number of replication origins will be much higher than the practically calculated number 300-400 origins. It suggests that a subset of replication origins are activated at a particular time and the others remain silent. If, it is true then focus should be given to those factors, which are responsible for it. Thus, the information collected from the various researches give mixed information about the real picture of DNA replication in yeast and need to be explored further in great detail.

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