INTRODUCTION

Soon after deoxyribonucleic acid (DNA) was proven as the genetic material (Hershey and Chase 1952), Watson and Crick proposed the double helical structure of DNA in 1953. The mechanism of duplication of the genetic material by unwinding of the double helix and copying the underlying sequence information was implicit in this model. The genome duplicates itself by a process termed as DNA replication within a short stretch of time, S phase, before cell division (cytokinesis) during the cell cycle. DNA replication is fundamental to life, and it is strictly regulated as it occurs only once per cell cycle (reviewed in Legouras et al. 2006). In order to preserve and faithfully transmit the genetic information through generations, different life forms have evolved various complex mechanisms ensuring strict regulation of DNA replication.

Jacob et al. (1963) for the first time tried to explain DNA replication of E. coli genome by proposing the replicon model. According to this model, DNA replication starts at a specific point or a cis-acting genetic element called as replicator with the help of trans-acting factors or regulatory proteins, called as initiators. Replicon was defined as a genetic element that replicated from a single origin of replication. This model was verified by extensive DNA sequencing and protein based studies (Bramhill and Kornberg 1988) but the duplication of genome in more complicated organisms could not be explained using this model.

In viral and prokaryotic genomes, replication initiates at a single, non-redundant, well-defined replicator or origin of replication (Kornberg and Baker 1992). Thus, these genomes function as a single replicon. A number of origins from viruses and prokaryotes are well defined now. For example, in bacteria (OriC of E. coli, origin of Bacillus subtilis), in 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 in 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).
On the other hand, eukaryotic genomes are made up of large and linear DNA molecules packed into chromosomes. Replication starts at multiple initiation sites (origins) throughout the length of the chromosomal DNA (Huberman and Riggs 1968) during S phase of the cell cycle. Among the eukaryotes, origins are best characterized in the budding yeast, *Saccharomyces cerevisiae* in which small, specific DNA sequences ranging from ~100 to 150 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 independently developed 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.*
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 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).

During the last decade, a number of microarray-based genome-wide origin analysis studies 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).

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).

Starting from the initial microinjection experiments in *Xenopus* egg nucleus (Harland and Laskey 1980; Mechali and Kearsey 1984) to different autonomous replication studies in animal cells, for example, the origin which is used to amplify the third chromosome chorion genes during *Drosophila* development (reviewed in Orr-Weaver 1991), the origin located downstream of the dihydrofolate reductase gene in CHO cells (Handeli et al. 1989), Lamin B2 (Giacca et al. 1994) and the origin responsible for replicating the human β-globin gene domain (Kitsberg et al. 1993), all suggest that initiation sites at animal cell origins are distributed in broad regions of several kilobase-pairs or larger called initiation zones (reviewed in Burhans and Huberman 1994).
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). 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).

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 \textit{ars3002}, the efficient ARS of the \textit{ura4} origin region on chromosome 3, has been reported (Kim and Huberman 1999). In 2004, Yompakdee and Huberman reported a sequence element associated with \textit{ars727} on chromosome 2 that appears to control the timing of initiation of the nearby ARS elements in plasmids. Interestingly, \textit{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 \textit{ars727} in chromosome 2 (Dubey \textit{et al.} 2010). These observations strongly suggested the possibility that the plasmid replication time determining elements associated with \textit{ars727} play a similar role in chromosomes too. To further explore this interesting possibility, I planned to make clones suitable for making necessary genomic alterations and preparing new \textit{S. pombe} strains for studying the role of the late replication enforcing sequences in determining the chromosomal replication timing. The objectives of the present study are:

1. Construction of clones for replacing \textit{ars2004} with \textit{ars727} (with and without late replication enforcing region) and \textit{vice-versa} in both orientations by multistep, PCR-based cloning. The \textit{ars2004} is an early firing efficient chromosomal origin, which fires in almost all the cell cycles (~90%) while \textit{ars727} is a late firing origin, which is chromosomally inactive but active as plasmid origin.

2. Functional analysis of these origins in their new context (flanking sequences) by yeast transformation assay (ARS assay) and monitoring cell cycle progression of the transformed cells.