REVIEW ARTICLE

Origins and complexes: the initiation of DNA replication

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Abstract

Eukaryotic DNA is organized for replication as multiple replicons. DNA synthesis in each replicon is initiated at an origin of replication. In both budding yeast, Saccharomyces cerevisiae and fission yeast, Schizosaccharomyces pombe, origins contain specific sequences that are essential for initiation, although these differ significantly between the two yeasts with those of S. pombe being more complex than those of S. cerevisiae. However, it is not yet clear whether the replication origins of plants contain specific essential sequences or whether origin sites are determined by features of chromatin structure. In all eukaryotes there are several biochemical events that must take place before initiation can occur. These are the marking of the origins by the origin recognition complex (ORC), the loading onto the origins, in a series of steps, of origin activation factors including the MCM proteins, and the initial denaturation of the double helix to form a replication ‘bubble’. Only then can the enzymes that actually initiate replication, primase and DNA polymerase-α, gain access to the template. In many cells this complex series of events occurs only once per cell cycle, ensuring that DNA is not re-replicated within one cycle. However, regulated re-replication of DNA within one cell cycle (DNA endoreduplication) is relatively common in plants, indicating that the ‘once-per-cycle’ controls can be overridden.

Key words: ARS, DNA polymerase, DNA replication, initiation, MCM, ORC, origin, primase, replicon.

Introduction

Plants, in common with all eukaryotic organisms, organize their DNA for replication as multiple units known as replicons (Van’t Hof, 1985, 1988). Each replicon is defined spatially by an origin of replication (ori) and two termini: the process of replication proceeds outwards from the origin to the termini. Whether the termini are specific sites or simply zones in which replication forks finally stall is not clear although evidence has been presented for specific termini in the replicons within the repeated genes that encode rRNA in pea (see below) (Hernandez et al., 1988b). However, it is clear that under circumstances in which fewer replication origins than usual are being utilized (see below) replication forks can continue through sites/zones which would in other circumstances be termini. Typically, the time taken to complete replication within one replicon is considerably shorter than the S-phase. For example, in pea it takes about 2 h to complete a replicon but S-phase may last for up to 8 h (Van’t Hof and Bjerknes, 1981). The explanation for this is that replicons are organized as time-groups or families with each family having its particular time within S-phase during which it is active in replication. This is most clearly seen in the small genome of Arabidopsis thaliana with only two replicon families, one of which completes replication in the first 2 h of S-phase whilst the second is delayed in initiation until 35–40 min after the start of S-phase, again taking about 2 h to complete replication (Van’t Hof et al., 1978). Clearly, the initiation of replication at the origins of the earliest replicon family represents the final stage in the transition from G₁ to S, but of course that initiation process must also occur within each replicon family as its time for activity arrives during S-phase. This is the setting for this brief review which will examine what is known about the biochemistry of initiation of replication in plants against a background of knowledge obtained in other eukaryotic organisms and particularly in budding

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yeast, *Saccharomyces cerevisiae* and fission yeast, *Schizosaccharomyces pombe*.

**Origins of replication**

**General features**

In plants the organization of DNA as multiple replicons can be readily visualized by fibre autoradiography (Van’t Hof, 1975, 1985, 1988), in which replicating DNA molecules are labelled with tritiated thymidine. Use of increasing labelling times facilitates the determination of ‘fork rate’, i.e. the rate of replication. ‘Step down’ labelling (transfer from high to low specific activity thymidine) shows the bidirectional movement of the forks and facilitates the identification of the origins. This in turn allows measurement of origin-to-origin distances (i.e. replicon lengths). In a given species, there is usually a clear mode of replicon length with some variation either side of the mode. Thus in pea root meristems the modal replicon size is about 54 kb (Van’t Hof and Bjerknes, 1977). However, there are well-defined situations in several plant species in which the replicon size shortens significantly, implying that more origins must be employed. This is discussed in more detail later in the review.

**What is an origin?**

Although replication origins may be ‘seen’ in fibre autoradiographs, it is much more difficult to separate a functional origin from the rest of the DNA; very few have been isolated from the DNA of multicellular eukaryotes. Many attempts to identify putative replication origins have relied on assessing the ability of DNA fragments to facilitate the so-called autonomous replication of plasmids in yeast (*Saccharomyces cerevisiae*) (Stinchcomb *et al.*, 1980). These pieces of DNA are known as ARS elements. This was originally coined as an abbreviation for **Autonomous Replication in Saccharomyces**, but now taken as meaning autonomously replicating sequence. Further, some authors use the term as being synonymous with replication origin. ARSs have indeed been shown by 2-D gel electrophoresis to act as *bona fide* origins in yeast itself, both in plasmids and in chromosomal DNA (Brewer and Fangman, 1987; Huberman *et al.*, 1988). The ability to isolate functional origins has also led to their characterization in terms of sequence: *ARS1* may be taken as a typical example. It consists of four sequence domains, A, B1, B2 and B3, all of which are needed for function (Marahrens and Stillman, 1992). The A domain is highly conserved between different origins and contains the absolutely essential core sequence, A/TTTTATG/ATTTA/T. All budding yeast replication origins have at least a 9/11 match to this sequence and will not function as an origin without it. The B domains, however, are more variable from origin to origin. The functional significance of this variation is not clear, but it is tempting to speculate that it may be related to the time of activation (often known as ‘firing’) of the origins within S-phase.

DNA sequences from many other eukaryotes, including plants, have been shown to function in the budding yeast ARS assay (Stinchcomb *et al.*, 1980). As might be expected from the essential nature of the ARS core sequence, all the higher eukaryote ARS elements that have been sequenced contain at least one essential core sequence. Indeed, as exemplified by ARS elements isolated from *Brassica napus* (Sibson *et al.*, 1988) some contain more than one match. What exactly does this say about DNA replication origins in higher eukaryotes? Firstly, it is inevitable that an assay depending on functioning as an origin in budding yeast will select for the sequences that yeast uses for origins. Secondly, in the large genomes of multicellular eukaryotes a sequence of 11 base pairs is very likely to occur by chance. The presence of budding yeast-type ARS elements in plant DNA does not therefore show that these elements are involved in replication origins in plants. Indeed, recent studies on origins of replication in the fission yeast *Schizosaccharomyces pombe* have indicated that *S. cerevisiae* may be a poor model for the structure of eukaryotic replication origins. These two yeasts are evolutionarily divergent and *S. pombe* ARS sequences (similarly identified by plasmid assay—but in *S. pombe*, not *S. cerevisiae*—and shown to be *bona fide* chromosomal origins) are significantly different in structure from those in *S. cerevisiae*. They are much larger (500–1500 bp as opposed to <150 bp for budding yeast) and their sequence composition is more complex, lacking a specific essential ARS core sequence. Instead they contain AT-rich 20–50 bp regions containing clustered A or T stretches, which are required for ARS activity (Clyne and Kelly, 1995; Dubey *et al.*, 1996; Kim and Huberman, 1998; Okuno *et al.*, 1999). Unlike budding yeast, *S. pombe* ARSs appear to map in promoter regions upstream from genes, suggesting that origin function may be related to transcription (Gómez and Antequera, 1999). Consistent with these differences, *S. pombe* ARSs do not normally function in *S. cerevisiae*, and vice versa (Clyne and Kelly, 1995). The exact extent of similarity between *S. pombe* ARSs and DNA replication origins in multicellular eukaryotes remains to be seen, but it is certainly clear that budding yeast origins differ in significant aspects from those in other eukaryotes.

Direct isolation of plant DNA replication origins has proved very difficult. However, multicellular eukaryotes possess an origin of DNA replication in each non-transcribed spacer (NTS) between the repeated genes that code for rRNA. The ‘bubbles’ associated with initiation at these sites are visible in electron micrographs of replicating DNA in *Drosophila*, *Tetrahymena* and *Xenopus* (reviewed by Van’t Hof, 1988). Van’t Hof’s group
(Van’t Hof et al., 1987a, b; Hernández et al., 1988a) have taken advantage of the high level of repetition of these genes in pea: in cultured pea root meristems they localized the site of initiation of replication to a 1500 bp region within the NTS. Subsequent 2-D gel experiments confirmed that the replication bubble that marks the initiation of strand separation occurs within this region (Van’t Hof and Lamm, 1992). Interestingly, the region contains a very AT-rich domain that includes four good matches to the S. cerevisiae ARS core (Hernández et al., 1988a, 1993).

On this basis, it appears that the budding yeast ARS core sequence may also be part of plant origins, despite the absence of this sequence in fission yeast origins (as discussed above). This suggestion is supported by the finding that replicative DNA isolated in very early S-phase in the same synchronized meristem system is enriched for similar sequences (Bryant, 1994). Further, the origins of replication from which the chorion genes in Drosophila are amplified are also AT-rich and contain sequences very similar to the ARS core (Austin et al., 1999; Spradling, 1999). However, some investigators have suggested that initiation of replication in higher eukaryotes has much ‘looser’ sequence requirements than in budding yeast (DePamphilis, 1993; Bogan et al., 2000), or even no sequence requirement at all (Mechali and Karsey, 1984; Gillespie and Blow, 2000). In reviewing this topic, Gilbert suggests that in animals, origins are localized to specific sequences not because of the sequences per se but by features of chromosome structure (Gilbert, 1998). This will be considered again in the context of origin recognition.

Understanding of the structure of plant DNA replication origins is further complicated by the findings that origin-to-origin spacing may vary in plant development, or in response to nutrients or hormones or to experimental manipulation. Thus in Sinapis alba, the floral stimulus induces in the shoot meristem a dramatic shortening of the S-phase that is mainly brought about by a halving of the modal replicon length from 15 kb to 7.5 kb, i.e. twice as many origins are utilized (Jacqmard and Houssa, 1988). Increase in the number of active origins during the transition to flowering also occurs in Silene coeli-roosa and Pharbitis nil (Durdan et al., 1998). In Sinapis, this aspect of the floral response may be mimicked by application of the hormone cytokinin (Houssa et al., 1990). Cytokinin has the same effect on dividing cells in the vegetative shoot apex of a grass, Lolium temulentum and in the ovule of tomato (Lycopersicon esculentum) (Houssa et al., 1994). In synchronized pea root meristems, cross-linking the DNA with psoralen in order to stall fork movement causes extra origins to be utilized between the cross-links (Francis et al., 1985). All these data suggest that plant DNA contains sequences that normally do not act as origins, but can be called into action under particular circumstances. By contrast, in lettuce roots, addition of trigonelline causes a halving of the number of active origins, with two out of every cluster of four being silenced or switched off (Mazzuca et al., 2000). In the Sinapis alba shoot meristem, abscisic acid caused a doubling of replicon length (15 kb to 30 kb: Jacqmard et al., 1995) implying that only one out of every two normally used origins was activated (as opposed to cytokinin which causes ‘extra’ origins to be activated, as described above). An earlier suggestion that is consistent with all these data was that plant DNA contains ‘strong’ and ‘weak’ origins (Francis et al., 1985). This may relate to the finding in S. pombe of clustered origins of replication which demonstrate hierarchies of initiation frequencies (Dubey et al., 1994; Okuno et al., 1997; Kim and Huberman, 1999). However, if ‘strength’ or ‘weakness’ is related to sequence, this does not explain the selective use of only one origin in six of those located in the non-transcribed spacers of the plant rRNA genes. Potential origins are 9 kb apart, but the modal replicon length is still 54 kb although the sequences of the NTSs are very similar. Van’t Hof speculates that nucleosome spacing may be an important factor in determining which of these origins is active (Van’t Hof, 1988). This suggestion is clearly compatible with Gilbert’s idea that initiation of replication at specific sequences is dependent on chromosome structure (as mentioned above) (Gilbert, 1998).

What happens at an origin?

Origins of replication are the places at which DNA replication starts. The general biochemical activities involved in this are self-evident: the origins must be recognized and activated and the synthesis of daughter strands must be initiated. These three activities are now considered.

Recognition of origins

The presence in budding yeast of replication origin sequences that are essential for function led, after some false starts, to the isolation of a complex of six proteins, the origin recognition complex (ORC) that binds to the ars core sequence of the A-domain in the origin (Bell and Stillman, 1992). The sequence specificity is based on the binding requirements of five out of the six subunits of the ORC (Lee and Bell, 1997). The role of the ORC is to ‘mark’ origins of replication and in budding yeast the ORC is bound to the origin for nearly the whole of the cell cycle (Diffl ey and Cocker, 1992; Dutta and Bell, 1997). However, although the ORC plays no direct role in origin activation or in initiation, the strict sequence requirements for ORC binding in budding yeast that directly parallel the strict sequence requirements for origin function (as described earlier) suggest that this marking of the origins by the ORC is essential for the initiation of DNA replication. Since it is now clear
that plants also possess ORC proteins (Gavin et al., 1995) it is legitimate to ask what happens in respect of origin recognition in those situations in which extra origins are used, as described above. Are these extra origins already ‘marked’, is more ORC synthesized or recruited or do plants have a less rigid requirement for the presence of the ORC in the initiation of replication?

This leads back to a consideration of whether plant and other higher eukaryotic origins are specific sequences. Unfortunately, the limited characterization of plant ORCs does not reveal anything about their sequence requirements. However, some data from Xenopus may be relevant here (Gillespie and Blow, 2000). A cell-free extract of Xenopus oocytes will replicate sperm DNA, added to the extract in the form of sperm chromatin. The oocyte extracts ‘sets up’ replication origins at 10 kb intervals by the binding of the ORC. In this system at least, the Xenopus ORC exhibits no specific sequence requirements and there is no specific sequence associated with the sites used as origins. This system is unusual (indeed, Xenopus embryo cells ‘revert’ to use of specific origins at the mid-blastula transition) but, nevertheless, the data raise the possibility that origin function in at least some higher eukaryotes may not always depend on specific sequences. By contrast, in recognizing the origin of replication within the chorion gene in Drosophila, the origin recognition complex shows a specificity for the 440 bp AT-rich tract referred to earlier (Austin et al., 1999). These data imply that origins may be at least partly defined by sequence. Clearly a more detailed functional analysis of ORCs in multi-cellular eukaryotes, including plants, is required.

**Activation of origins**

In order to set the scene for this topic it is necessary to discuss data from budding yeast and from fission yeast. In budding yeast, the region of the origin protected by the ORC (as defined by DNase protection footprinting) is extended, from late anaphase and throughout the G1 phase, by the binding of additional components (Diffley et al., 1994; Cocker et al., 1996). The increase in the footprint is due to the binding of an ‘origin loading factor’ (see below) followed by the binding of an additional protein complex known as the MCM complex, consisting of six different MCM proteins (MCMs 2-7), (so named because they were discovered as being the products of genes essential for mini-chromosome maintenance in yeast). The binding of the MCM complex (or, more probably, two complete MCM complexes: see below) actually requires the prior recognition of an ORC-marked origin by the loading factor (Cocker et al., 1996; Kearsey et al., 2000). In fission yeast and vertebrates the loading factor consists of two proteins acting together, namely Cdc6/cdc18 and cdt1 (Maiorano et al., 2000; Nishitani et al., 2000) (Fig. 1). In budding yeast, only Cdc6 appears to be involved at this point (Cocker et al., 1996). Both Cdc6/cdc18 and cdt1 are regulated by transcription during G1 (Drury et al., 1997; Lopez-Girona et al., 1998). The dissociation of the Cdc6/cdc18 protein from the pre-replicative complex (in fission yeast this is driven by a cdc2-kinase-mediated phosphorylation: Lopez-Girona et al., 1998) and its subsequent degradation (Drury et al., 1997) all contribute to the control system ensuring that in a normal cell cycle, each origin is only fired once. Further, in budding yeast, the activity of the MCM complex appears to be dependent on the dissociation of the Cdc6 protein from chromatin (Hua and Newport, 1998). The MCM complex also interacts with Mcm10/cdc23 (S. cerevisiae/S. pombe) which is essential for correct DNA replication (Merchant et al., 1997; Aves et al., 1998; Homesley et al., 2000). The MCM complex is then phosphorylated by the Cdc7-Dbf4 protein kinase, without which the complex cannot

![Fig. 1](image_url)
activate the origin. Following this, the MCM proteins are joined by the protein encoded in budding yeast by \textit{CDC45}, the role of which is to facilitate the loading of the initiation complex (Mimura and Takisawa, 1998; Tye, 1999; Walter and Newport, 2000). This sequence of events is summarized diagrammatically in Fig. 1.

So, what is the role of the MCM complex? MCM proteins contain a sequence domain that indicates the possession of DNA helicase/DNA-dependent ATPase activity (Koonin, 1993). Direct biochemical demonstration of this helicase activity in individual MCM proteins is difficult and current evidence suggests that the activity is associated with a sub-complex consisting of MCM4, 6 and 7 (Ishimi, 1997). Rather confusingly, MCM2 appears to inhibit this helicase activity (Ishimi, 1997). Interestingly, evidence from the archaeon, \textit{Methanobacterium thermoautotrophicum}, in which a single MCM gene is present, shows that the protein exists both as a monomer and as a double hexamer complex (Kelman et al., 1999) with both a DNA-dependent ATPase activity and 3’ to 5’ helicase activity sufficient to unwind 500 base pairs of DNA (Kelman et al., 1999; Chong et al., 2000; Shechter et al., 2000). Although the archaeal system is considerably simpler than that of eukaryotes, these results suggest that the MCM complex may act as the replicative DNA helicase in eukaryotes and archaea (but see below).

Features that are clear are that the activity of the MCM complex firstly displaces, at least temporarily, the ORC from the origin and secondly allows Cdc45-mediated access to the template of the initiation complex, including primase and DNA polymerase-\(\alpha\). This implies that extensive enough strand separation has occurred to allow the formation of a replication bubble. Further evidence that it is the helicase activity of the MCM complex that separates the two strands comes from findings that in budding yeast the two MCM complexes remain associated with or even generate the two moving replication forks, consistent with their DNA helicase activity referred to above (Aparicio et al., 1997; Tye, 1999). However, this may not be true of all eukaryotes: in \textit{Xenopus}, for example, MCMs are displaced from chromatin after replication is initiated (Coué et al., 1996). Further, in animals (Wang, 1996) and plants (JA Bryant, unpublished data) a separate helicase enzyme is part of the multi-protein complex associated with DNA polymerase-\(\alpha\)-primase (see below). The presence of both this helicase and at least one of the MCM proteins makes it unclear as to which of these is involved in separating the DNA strands to generate the replication forks. It may of course be both.

The situation in plants is, in general, less well-defined than that described above for budding yeast and for fission yeast. Homologues of the \textit{CDC6} and \textit{cdt1} genes have been detected in plants (Lin et al., 1999; Nishitani et al., 2000; Whittaker et al., 2000) and their sequences are similar enough to those of budding yeast and/or fission yeast to indicate a similar function. It is also clear that plants possess all the members of the MCM complex, as detected by immunological screening, from cDNA or gene sequences and by protein purification (Ivanova et al., 1994; Springer et al., 1995; Sabelli et al., 1996; Moore et al., 1998; Munns et al., 1998). The gene sequences are again similar to those of their homologues in yeast, and, for each member of the MCM complex, are very similar between different plants. For example, the sequence of MCM3 from pea is, at the amino acid level, 69% identical to that from maize, 67% identical to \textit{Arabidopsis}, and 51% identical to the human MCM3 (Moore et al., 1998). This is a very high level of sequence conservation and implies an essential function right across the eukaryotes. One slight puzzle, however, is that although the sequences of plant \textit{MCM} genes may be translated to give protein sequences of similar lengths to those of other eukaryotes, the MCM proteins that have been detected all appear to be smaller on the basis of their mobility in denaturing polyacrylamide gels. For example, the pea MCM3 appears to have an \(M_r\) of c. 66 kDa (Munns et al., 1998) whereas the size and sequence of the mRNA suggests a protein of about 100 kDa. The reason for this discrepancy is not known at present although it is possible either that the plant MCMs are subject to partial proteolysis or that they behave anomalously in polyacrylamide gels.

Although the ‘behaviour’ of plant MCM proteins on the DNA template has not been established, it is clear that they are nuclear-located (Ivanova et al., 1994; Sabelli et al., 1999). Further, a recent immunological study with the maize MCM3 indicates that the intra-nuclear location of the protein changes during the cell cycle in a manner that is at least consistent with an association with chromatin for part of the cycle (Sabelli et al., 1999). However, any dependence of such a binding on the ORC or on a Cdc6 homologue has yet to be demonstrated. One further point about plant MCMs is that an MCM3-like protein has been detected in the primase-polimerase-\(\alpha\) complex (Munns et al., 1998), i.e. the replication initiation complex (see below). For mammals, the first purification of an MCM protein was that of MCM3 from the human primase-polimerase-\(\alpha\) (Thommes et al., 1992) and it raises the possibility that MCM3 may have a direct role in loading the initiation complex on to the activated origin.

### Initiation of DNA synthesis

Of the multiple DNA polymerases that occur in eukaryotes, only one is able to initiate the synthesis of new strands. This is DNA polymerase-\(\alpha\), the initiating ability of which resides in its close association with DNA primase (Foiani et al., 1997). The latter is in effect
a very specialized RNA polymerase that lays down oligo-ribonucleotide primers of about 11 bases on the single-stranded DNA template generated by the activation of the replication origins and the subsequent outward movement of the replication forks. The details of this process have been elucidated with a model system for eukaryotic DNA replication, namely the replication of a viral DNA, SV40 (Waga and Stillman, 1994, 1998). In this system, the activation of the viral origin of replication and at least the initial separation of the parental DNA strands is brought about by a virus-encoded protein, the T-antigen. However, all subsequent steps in replication are mediated by the host cell (i.e. monkey or human) enzymes. The first step is the laying down of the primers for the leading strands; the primers are then extended a short distance by polymerase-α. However, the polymerase-α very quickly ‘hands over’ to the much more processive DNA polymerase-δ that mediates the bulk of daughter strand synthesis (details of the latter lie outside the scope of this review which is focused on initiation). At this point, the primase–polymerase-α complex switches strands and becomes responsible for the initiation of every Okazaki fragment on the lagging strand (Fig. 2).

Although the details of initiation were worked out with the SV40 model system, there is extensive biochemical and genetic evidence from a range of eukaryotes to confirm the role of primase–polymerase-α as the initiating enzyme complex. Focusing specifically on plants, DNA polymerases of the α-type have been extensively purified from several plant species including pea (Stevens and Bryant, 1976; Bryant et al., 1992), wheat (Litvak and Castroviejo, 1985) maize (Coello and Vazquez Ramos, 1995), rice (Sala et al., 1981), and spinach (Misumi and Weissbach, 1982). The pea polymerase-α has an associated primase activity that is able to use ribonucleotides to synthesize primers on single-stranded M13 bacteriophage DNA; these may then be extended by the polymerase itself (Bryant et al., 1992). Purification of the pea primase has so far proved impossible because the enzyme loses activity on separation from the polymerase. However, the tobacco (Garcia Maya and Buck, 1998) and wheat (Laquiel et al., 1994) primases have been purified and the specific primase activity of the latter has been demonstrated both on its own and in add-back experiments with DNA polymerase-α.

Primase and DNA polymerase-α are obviously closely associated. Nevertheless, there is some evidence that the polymerase needs assistance in locating the primers laid down by its associated primase activity. In pea, a DNA-binding protein that initially co-purifies with polymerase-α (Al Rashdi and Bryant, 1994) has been shown to bind strongly to ds-ss junctions such as would be generated at the 3′-OH terminus of a primer (Burton et al., 1997). In add-back experiments with primase–polymerase-α, the DNA-binding protein stimulates significantly the polymerase activity when the polymerase is working on M13 DNA templates that have been primed by the associated primase activity or by the addition of a single sequencing primer. It does not stimulate polymerase-α when the latter is working on a template with very closely spaced primers, such as ‘gapped’ DNA (Bryant et al., 2000). A very similar primer-recognition protein has also been purified from human cells (Jindal and Vishwanatha, 1990).
where its role has been described as ensuring ‘productive’ binding of the polymerase to the template adjacent to the 3’-OH primer terminus. However, such an activity does not feature in the details of initiation worked out for the SV40 model system (Waga and Stillman, 1994, 1998) and it remains to be seen whether this is a universal feature of the initiation of DNA synthesis in eukaryotes.

Once and once only

In a ‘normal’ cell cycle, the processes described above occur just once for each replicon; DNA replication is completed, but is not re-initiated. Detailed discussion of this lies outside the scope of this review. It is, however, interesting to note that several different levels of control may be envisaged. At the level of origin activation, Laskey and his associates have proposed that at least in vertebrates, origins must be licensed by a specific factor that either leaves the nucleus or is degraded after initiation and that a new population of licensing factor may only gain access to the origins after breakdown of the nuclear envelope during mitosis. There is in fact some good evidence for such a factor in vertebrates (Coverley and Laskey, 1994; Thömmes and Blow, 1997; Donaldson and Blow, 1999), but not yet in other eukaryotes. Indeed, for yeasts, which undergo a closed mitosis, this hypothesis is clearly not applicable in detail. In respect of the yeasts, the inactivation and turnover of the MCM loading factors (Cdc6 in budding yeast and cdc18 plus cd1 in fission yeast) have already been noted as has the cell-cycle-phase-dependent transcription of the corresponding genes. A control based on the availability of these loading factors may therefore be envisaged. Some support for this view comes from the finding that over-expression of cdc18 in fission yeast does indeed lead to extra rounds of DNA replication in the absence of mitosis (Lopez-Girona et al., 1998) (although over-expression of Cdc6 in budding yeast does not: Drury et al., 1997). However, the most widely applicable hypothesis is that the normal fluctuations of the cyclin-dependent kinases, determining entry into S and into M, coupled with the operation of checkpoints, is enough to ensure that DNA is replicated only once per cell cycle (Larkins et al., 2001). For example, the ability of MCMs to bind to chromatin may be modified by phosphorylation by cdks. The G1 and s cdk–cyclin complexes (cdk–cyclin-A and cdk–cyclin-E) phosphorylate MCMs at a low level and this permits the MCMs to bind to chromatin. However, chromatin binding is very much reduced or even abolished when the MCMs are phosphorylated by cdk–cyclin-B, the kinase that drives the cell through G2/M. Destruction of cyclin-B in mitosis is therefore required to permit MCM binding in order to allow DNA replication in the next cell cycle. None of this of course precludes the possibility of other levels of regulation such as other licensing factors that may associate with the MCMs, although recent data from plants and mammals certainly point to the central role of the cyclin-dependent kinases even in complex multi-cellular eukaryotes (Larkins et al., 2001; Hattori et al., 2000).

In plants, there are many cell types in which DNA endoreduplication (re-replication of DNA without an intervening mitosis) occurs in a highly regulated way as part of cellular differentiation. Indeed, Larkins and his colleagues (Larkins et al., 2001) suggest that endoreduplication is so common in plants that it should not be thought of as an abnormal cell cycle, but simply a commonly occurring variant of the cell cycle. The same authors conclude from their own work and those of others that endoreduplication may be achieved by a loss of the cyclins and cyclin-dependent kinases that drive the cell into M-phase. If a background of cdk–cyclin D expression (Riou-Khamlichi et al., 1999) is maintained in order to keep the cells potentiated for division (Frank and Schmülling, 1999), then the normal cell-cycle-based fluctuations in the activity of the A and the E cyclins (plus their corresponding kinases) will be adequate to drive the cell into repeated S-phases, provided that the relevant checkpoints may be bypassed (Larkins et al., 2001). Some evidence that is consistent with this view comes from the results of Jacqmard et al. who showed that cyclin-B was present in Arabidopsis cells undergoing the ‘normal’ mitotic cell cycle, but not in cells that were endoreplicating their DNA (Jacqmard et al., 1999).

Concluding comments

The ability to replicate the genetic material is the most fundamental aspect of living cells. The essential physico-chemical features of the process were deduced from DNA structure nearly 50 years ago (Watson and Crick, 1953) and confirmed by the elegant experiments of Meselson and Stahl five years later (Meselson and Stahl, 1958). Yet the detailed biochemistry of replication is still being worked out, especially in eukaryotes. It remains a major and exciting challenge in this age of genomics to reach a fuller understanding of the mechanisms and of their control and then, perhaps even more challenging, to understand how these processes are integrated within plant development. There is still much to do.

Note added in proof

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