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Roles of Phosphorylation in DNA Replication

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Phosphorylation of proteins by protein kinases and dephosphorylation by protein phosphatases represents one of the most common, versatile, and perhaps confusing regulatory mechanisms in eukaryotic cells. Many, perhaps most, proteins in the eukaryotic nucleus are phosphoproteins, among them the proteins involved in DNA replication and its control. The importance of protein phosphorylation as a regulatory mechanism lies in its ready response to intracellular or extracellular signaling, its reversibility, and its ability to act as a measuring device to translate gradual changes into a molecular switch thrown at a threshold level of phosphorylation of a key target protein. The net level of protein phosphorylation is determined by the balance between the activities of protein kinases and those of protein phosphatases (for review, see Cohen 1989; Hubbard and Cohen 1993). The activities of kinases and phosphatases themselves are regulated by their own phosphorylation state. To complicate matters further, the number of known protein kinases, phosphatases, and regulatory subunits is growing rapidly, and their specificity is not always predictable from the primary sequence of the substrate protein (see Moreno and Nurse 1990; Cegielska et al. 1994a). Moreover, the effect of phosphorylation on the activity of a given target protein usually depends on the exact sites that are modified and unmodified. Thus, to understand how protein phosphorylation regulates the activity of replication proteins, one must know which sites in a target protein are phosphorylated, how physiological signals affect phosphorylation at each site, and how this phosphorylation influences the protein's activity. Knowledge of protein kinases or phosphatases that act at each phosphorylation site is also useful in elucidating the role of protein phosphorylation in regulating the target protein.

The role of protein phosphorylation in control of the timing of DNA replication in the cell cycle has been studied extensively (for review, see Norbury and Nurse 1992; Coverley and Laskey 1994), and these phosphorylation events, as well as the cyclins, cyclin-dependent kinases, and their effects on substrates such as the retinoblastoma tumor suppressor protein are discussed by Nasmyth (this volume). Here we discuss specific examples of DNA replication proteins whose regulation by phosphorylation has been described in detail, summarize our current understanding of the regulation of several cellular replication proteins by phosphorylation, and, finally, speculate how phosphorylation could control the activity of some DNA replication proteins in the cell cycle.

PROTEINS THAT RECOGNIZE AND ACTIVATE REPLICATION ORIGINS

Simian Virus 40 Large Tumor Antigen

The replication of SV40 DNA is a useful model for eukaryotic DNA replication (see Brush and Kelly; Hassell and Brinton; both this volume). SV40 DNA is double-stranded, circular, and supercoiled. In infected monkey cells and in the virus particle, viral DNA is complexed with cellular histones, forming nucleosomes that resemble those in cellular chromatin (for review, see Fanning and Knippers 1992). A set of ten human replication proteins that is sufficient to replicate SV40 DNA in vitro has been purified and characterized (Waga et al. 1994; see Brush and Kelly; Hassell and Brinton; both this volume). Many of these mammalian proteins are functionally conserved in yeast, and the genes encoding many of them have been shown to be essential for viability in yeast (for discussion of these proteins, see Stillman; Wang; Coen; Hübscher et al.; Borowiec; Nash and Lindahl; all this volume), providing strong support for the validity of the SV40 model system and arguing that mammalian chromosomes are probably replicated by the same set of cellular proteins. Like mammalian chromosomal DNA, SV40 DNA replicates in the nucleus during the S phase in the cell cycle. It is not clear how SV40 DNA replication is limited to the S phase, but it is evident that cell-cycle-dependent protein kinases, phosphatases, and their regulatory subunits play an important role. However, unlike mammalian chromosomes, SV40 chromatin undergoes multiple rounds of replication during S phase, indicating that SV40 somehow circumvents the cellular mechanisms that limit chromosomal replication to a single round per S phase. Thus, viral replication is subject to some of the cellular controls on DNA replication, but not all of them.

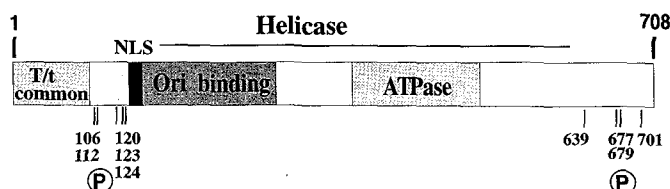
The only viral protein needed for SV40 DNA replication *in vitro* and in cultured monkey cells is the large tumor antigen (T antigen). T antigen is a multifunctional phosphoprotein whose intrinsic biochemical activities include sequence-specific binding to the SV40 origin of DNA replication, ATP binding and hydrolysis, and 3'-5' DNA helicase activity (for review, see Fanning 1992; Fanning and Knippers 1992). T antigen resides primarily in the nucleus of infected cells, where it serves to stimulate the cell to progress to the S phase prior to viral DNA replication and controls expression of viral and cellular genes and virion assembly, in addition to participating directly in viral DNA replication.

A basic understanding of the process of SV40 DNA replication and the action of the proteins involved has been developed through work performed using cell-free systems (for review, see Kelly 1988; Stillman 1989; Borowiec et al. 1990; Hurwitz et al. 1990). In the current model, SV40 T antigen binds specifically to sequences in duplex origin DNA and assembles in the presence of ATP into a double hexamer on the origin, causing local distortion or melting in the origin DNA sequences flanking the T-antigen-binding site (see Borowiec, this volume). In the next step of the reaction, the DNA helicase activity of the double hexamers, driven by ATP hydrolysis, bidirectionally unwinds the two parental strands by reeling the DNA through the complex (Dean et al. 1992; Wessel et al. 1992). The single-stranded regions are stabilized by the single-stranded DNA-binding protein RP-A, and the enzyme responsible for initiation of synthesis, DNA polymerase- α :primase (pol- α :primase), joins the protein-DNA complex. The assembly of this complex is directed by specific protein-protein interactions that occur among T antigen, RP-A, and pol- α :primase (Dornreiter et al. 1990, 1992, 1993; Melendy and Stillman 1993; Murakami and Hurwitz 1993; for review, see Diffley 1992). After synthesis of the first primer and Okazaki fragment, replication factor C (RF-C) displaces pol- α :primase from the primer-template junction and recruits proliferating cell nuclear antigen (PCNA). The RF-C/PCNA complex then targets DNA polymerase- δ to the junction, where it takes over leading-strand DNA synthesis (Lee et al. 1991a; Tsurimoto and Stillman 1991a, b, c). On the lagging strand, pol- α :primase initiates a new RNA primer, extends it into a short DNA primer, dissociates and then repeats this process to generate the next DNA primer (see Salas et al., this volume). To extend the DNA primers on the lagging strand into Okazaki fragments, polymerase δ , again with the help of RF-C and PCNA, binds to the termini and extends the fragments until it encounters the previous Okazaki fragment. RNase H, together with maturation factor I (MFI), removes the RNA primer, and

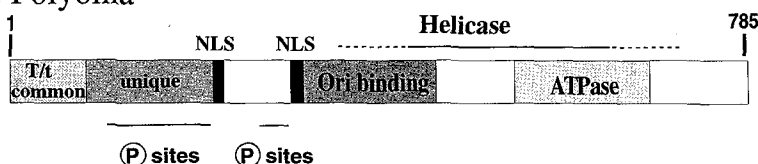
ligase I joins the two fragments (Turchi et al. 1994; Waga and Stillman 1994; Waga et al. 1994; see Brush and Kelly, this volume). Topoisomerases I and II release the torsional stress caused by the progression of the replication fork, and topoisomerase II also functions in the resolution of catenated replication products (Yang et al. 1987; Ishimi et al. 1988).

The replication functions of SV40 T antigen are tightly controlled by its phosphorylation state (for review, see Prives 1990; Fanning 1992, 1994). T antigen expressed in mammalian cells is phosphorylated on multiple serine (S) and threonine (T) residues clustered at the carboxyl and amino termini of the protein (Fig. 1A) (Scheidtmann et al. 1991). Mutational analysis of the T antigen phosphorylation sites in cell culture demonstrated that phosphorylation at six of these sites (S106, S112, S639, S677, S679, and T701) was dispensable, whereas T-antigen mutants bearing alanine in place of S120, S123, or T124 were defective

A. SV40



B. Polyoma



C. BPV

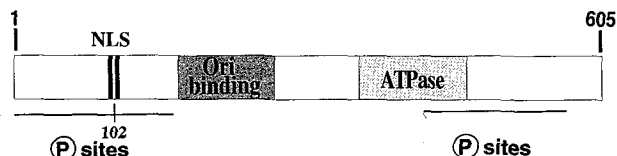


Figure 1 Functional domains and known phosphorylation sites of viral origin recognition proteins. (A) SV40 T antigen; (B) polyoma T antigen; (C) bovine papillomavirus E1 protein. The data were compiled from Fanning (1994); E.H. Wang et al. (1993); Lambert (1991); and unpublished data kindly provided by Mike Lentz. Phosphorylation sites (P) are indicated below each polypeptide by residue numbers (A), and regions containing phosphorylation sites by lines (B, C).

in viral replication *in vivo* (Schneider and Fanning 1988), suggesting that modification of these sites is required for SV40 DNA replication in monkey cells.

The role of phosphorylation of T antigen at these three sites in the control of viral DNA replication has been further investigated in cell-free reactions using purified proteins. These studies confirm the requirement for phosphorylation of T124 that was observed *in vivo* but suggest a quite different role for modification of S120 and S123, namely, that it inhibits viral DNA replication *in vitro*. For example, the bulk of the T antigen isolated from SV40-infected mammalian cells is hyperphosphorylated and inactive in SV40 DNA replication *in vitro* (Fig. 2) (Virshup et al. 1989). T antigen expressed in bacteria is not modified at these sites and is also unable to initiate SV40 DNA replication *in vitro* (Fig. 2) (McVey et al. 1989). However, T antigen expressed in recombinant baculovirus systems has an intermediate level of phosphorylation, with T124 modified in nearly all molecules, but with most serine sites remaining unmodified (Höss et al. 1990); this T antigen is highly active in SV40 replication *in vitro* (Fig. 2).

In agreement with these findings, treatment of T antigen with protein kinases and phosphatases that alter the phosphorylation state of T124, S120, or S123 activates or inhibits its replication activity *in vitro*. Phosphorylation of T124 by cdc2-cyclin B activated the replication activity of unphosphorylated bacterially expressed T antigen (McVey et al. 1989). The positive effect of phosphorylation of T124 on the replication activity of T antigen was reversed by treating it with isoforms of protein phosphatase PP2A that specifically targeted T124, i.e., PP2A-D and PP2A-T55 (Cegielska et al. 1994b). Treatment of hyperphosphorylated T antigen from mammalian cells with the catalytic subunit of PP2A (C36, PP2A_C) or with an isoform of PP2A (PP2A-T72) stimulated SV40 replication by dephosphorylating S120 and S123 (Virshup et al. 1992; Cegielska et al. 1994b). More recently, an isoform of casein kinase I was shown to phosphorylate these serines *in vitro* (Cegielska and Virshup 1993; Cegielska et al. 1994a). Kinase treatment of hypophosphorylated replication-active T antigen isolated from baculovirus-infected insect cells led to inactivation of SV40 replication activity. Taken together, these results indicate that SV40 DNA replication *in vitro* is regulated positively and negatively by the phosphorylation state of T antigen (Fig. 2).

The biochemical mechanism by which phosphorylation of T antigen regulated its replication activity was elucidated by comparing the activities of baculovirus-expressed wild-type and mutant T antigens.

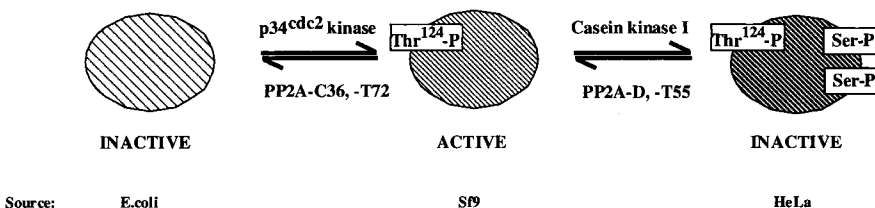


Figure 2 Phosphorylation controls SV40 T-antigen replication activity in vitro. Unphosphorylated T antigen synthesized in bacteria is inactive in replication unless it is first phosphorylated on Thr-124. Hypophosphorylated T antigen expressed in insect cells using baculovirus vectors is active, whereas hyperphosphorylated T antigen expressed in mammalian cells is inactive. Protein kinases and phosphatases (Cegielska et al. 1994a,b) that act on the crucial Thr-124, Ser-120, and Ser-123 sites are indicated. (Reprinted, with permission, from Fanning 1994.)

Phosphorylation at T124 is necessary for the bidirectional unwinding of duplex SV40 origin DNA by T antigen in the initiation reaction (McVey et al. 1993; Moarefi et al. 1993b). In contrast, other enzymatic activities like hexamer assembly, unidirectional DNA helicase, DNA binding, and origin DNA distortion were not markedly influenced by phosphorylation of T124. The specific effect of phosphorylation on bidirectional unwinding appears to result from its ability to stabilize hexamer-hexamer contacts that are necessary for bidirectional, but not unidirectional, DNA unwinding (K. Weisshart and E. Fanning, unpubl.; T. Kelly, pers. comm.). Phosphorylation of T124 also results in cooperative assembly of the two hexamers on the SV40 origin (K. Weisshart and E. Fanning, unpubl.; T. Kelly, pers. comm.).

Interestingly, phosphorylation of T antigen on the inhibitory serine residues also decreased the cooperative assembly of T antigen double hexamers on SV40 origin DNA, blocking bidirectional origin-unwinding activity and viral DNA replication in vitro (Virshup et al. 1992; Cegielska and Virshup 1993; Cegielska et al. 1994a). Thus, T antigen unphosphorylated on T124 or phosphorylated on S120 and S123 could potentially bind to the origin as a double hexamer and distort it locally without initiating bidirectional unwinding and replication until it became phosphorylated on T124 and dephosphorylated on S120 or 123, thereby facilitating interactions between the two hexamers that are required for bidirectional unwinding (Fig. 3). We suggest that the interactions between the hexamers are dynamic, undergoing cycles of association and dissociation as unwinding proceeds (Fig. 3). Although this mechanism

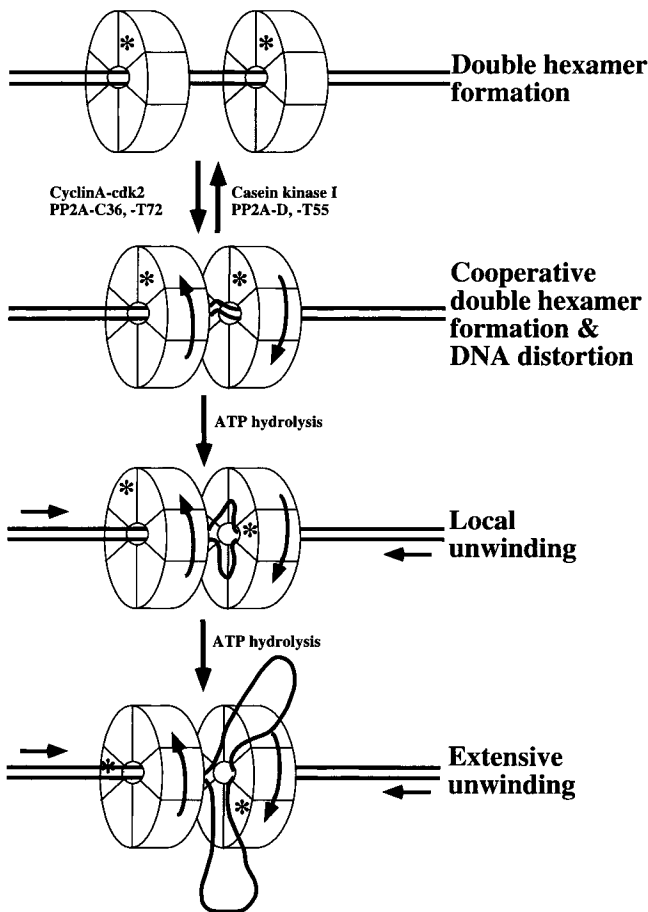


Figure 3 The phosphorylation state of SV40 T antigen governs hexamer-hexamer interactions in bidirectional origin unwinding. Prior to viral DNA replication in infected mammalian cells, T antigen accumulates primarily in hyperphosphorylated forms that can assemble as a double hexamer on the SV40 origin, but the hexamers cannot interact properly with each other to unwind the origin and permit initiation. Replication-active hexamers can be formed upon dephosphorylation of Ser-120 or Ser-123, or by phosphorylation of newly synthesized unphosphorylated T antigen on Thr-124. When a sufficient concentration of this hypophosphorylated form of T antigen is reached in the infected cell, ATP hydrolysis and dynamic hexamer-hexamer interactions lead to bidirectional unwinding at the origin and initiation of replication. Subsequent phosphorylation of Ser-120 and Ser-123 or dephosphorylation of Thr-124 would block reinitiation of replication. (Reprinted, with permission, from Fanning 1994.)

remains speculative, the data demonstrate clearly that the phosphorylation state of T antigen controls SV40 DNA replication both negatively and positively at the level of unwinding of origin DNA. It will be interesting to see whether phosphorylation of cellular origin recognition or unwinding proteins regulates their function in a similar manner.

The significance of T antigen phosphorylation in viral DNA replication *in vivo* remains for the most part unresolved. Although it is well established that phosphorylation of T124 is essential for SV40 DNA replication *in vivo* (Schneider and Fanning 1988), this modification may play additional roles in regulation of replication. One attractive model proposes that phosphorylation of T antigen at T124 could link the replication activity of T antigen to the cell cycle (McVey et al. 1989, 1993). T antigen is associated with cdk2-cyclin A in the cell (Adamczewski et al. 1993) and is a much better substrate for this kinase than for cdc2-cyclin B *in vitro* (C. Voitenleitner et al., unpubl.). cdk2-cyclin A is activated at the G₁-S transition and is found associated with replicating SV40 DNA (Fotedar and Roberts 1991). Together these data suggest a role for cdk2-cyclin A as the best candidate for phosphorylation of T124 *in vivo* and support the notion that this modification event could link SV40 DNA replication to S phase. However, the phosphate turnover on this site is relatively slow in SV40-infected cells, and most T-antigen molecules carry the modification (Scheidtmann 1986; for review, see Fanning and Knippers 1992). Furthermore, there is no direct evidence to suggest that modification of T124 in SV40-infected or -transformed cells is cell-cycle-dependent. Thus, it remains an open question whether the cell-cycle-dependence of SV40 DNA replication is controlled by this phosphorylation event.

The physiological role of phosphorylation of T-antigen residues S120 and S123 *in vivo* is even more puzzling. Whereas phosphorylation of S120 and S123 inhibits viral DNA replication *in vitro*, phosphorylation at these sites is required *in vivo* (Schneider and Fanning 1988). Reexamination of the kinetics of SV40 DNA replication in cultured cells with the S120A and S123A mutants yielded preliminary evidence that the mutant T antigens did begin viral DNA replication, but only a small amount of DNA accumulated (Moarefi et al. 1993a). To resolve the apparent discrepancy between the *in vivo* and *in vitro* results, it has been suggested that phosphorylation of these serine residues is necessary for regulatory functions not detectable in the *in vitro* replication system (Fanning 1994). Support for this hypothesis comes from the finding that phosphate turnover on these serines is rapid (Scheidtmann 1986), so that the balance between phosphorylation and dephosphorylation on these

two serines could determine the fraction of T antigen active in initiation of DNA replication (Fanning 1992). Phosphorylation at these serines may be required in the termination of replication, or it could influence the association of viral DNA with replication foci (Hozak and Cook 1994). These or other mechanisms might reduce the ability of T antigen to sustain multiple rounds of viral DNA replication in vivo, resulting in the defect observed with the S120 and S123 mutants in cell culture but not in vitro (Schneider and Fanning 1988; Moarefi et al. 1993a). Further work is required to resolve this paradox.

The physiological relevance of PP2A and casein kinase I action on T antigen in vivo also remains obscure. Casein kinase I is ubiquitously expressed and not regulated in the cell cycle. In addition, PP2A is found primarily in the cytoplasm rather than in the nucleus. However, one cannot exclude the possibility that the different isoforms of kinase and phosphatase that affect T antigen have different subcellular localizations. Indeed, there is evidence that the activity of PP2A on T antigen is restricted to S phase (Ludlow 1992). Other protein phosphatases that act at the G₁-S border may also target T antigen, such as a mammalian homolog of SIT4 from budding yeast and *Drosophila* (Fernandez-Sarabia et al. 1992; Mann et al. 1993) or a mammalian homolog of the yeast cdc25A phosphatase (Hoffmann et al. 1994; Jinno et al. 1994).

Polyomavirus Large Tumor Antigen

The initiator protein for polyomavirus (PyV) replication is the polyoma large T antigen, Py T, which interacts with specific sequences within the PyV origin of replication (Cowie and Kamen 1984; Hassell and Brinton, this volume). Like its SV40 counterpart, Py T antigen unwinds duplex DNA (Wang and Prives 1991) using its ATPase/helicase function (Seki et al. 1990). Py T antigen is a phosphoprotein with two clusters of phosphorylation sites located at the amino terminus (Fig. 1B) (Hassauer et al. 1986; Bockus and Schaffhausen 1987). No assessment of the importance of single phosphorylation sites has been undertaken, but treatment with calf intestinal phosphatase had differential effects on Py T antigen, depending on the degree of phosphate removal (E.H. Wang et al. 1993). Treatment with limiting amounts of phosphatase stimulated origin binding, whereas incubation with high concentrations of phosphatase had the opposite effect. These data are reminiscent of early results obtained with partially dephosphorylated SV40 T antigen (Grässer et al. 1987) and suggest that the replication activity of PyV T antigen might be regulated through differential phosphorylation. It will be interesting to test whether

phosphorylation of Py T regulates initiation of viral DNA replication by controlling the ability of Py T to catalyze bidirectional unwinding of the origin DNA as in the SV40 system.

Papillomavirus E1 Protein

Two bovine papillomavirus (BPV)-encoded proteins are necessary and sufficient for BPV DNA replication *in vivo*, the 68-kD E1 protein and the 48-kD E2 protein (for review, see Lambert 1991; Ustav and Stenlund 1991; Ustav et al. 1993; Stenlund, this volume). The E2 protein can be dispensable *in vitro* when high levels of E1 are used (Seo et al. 1993b; Spalholz et al. 1993). All other replication proteins are supplied by the host cell. E1, like SV40 and Py T antigens, is a phosphoprotein that possesses several activities necessary for replication function (Ustav and Stenlund 1991; Seo et al. 1993a; Thorner et al. 1993; Yang et al. 1993; Park et al. 1994; Bonne-Andrea et al. 1995): origin binding activity, polymerase- α binding, ATPase activity, and 3'-5' helicase activity (Fig. 1C). E2 protein is a transcriptional activator (Spalholz et al. 1993) and also a phosphoprotein (Bream et al. 1993) that stimulates the replication activity of E1 protein *in vitro*, at least in part by binding to it (Seo et al. 1993b; Spalholz et al. 1993; Benson and Howley 1995). It has been proposed that the role of E2 is to tether E1 more tightly to the origin and to recruit cellular replication factors, e.g., RP-A and pol- α :primase (Li and Botchan 1993), to the BPV origin.

The facts that BPV DNA is maintained as an episome at a constant copy number in latently transformed cells (Law et al. 1981) and that it replicates during S phase along with the host chromosomal DNA (Gilbert and Cohen 1987) might suggest that the activities of E1 and E2 are regulated in the cell cycle. Since both proteins are phosphoproteins, this regulation could depend on phosphorylation events. The E1 residue T102 near the nuclear localization signal is a substrate *in vitro* for cdc2-cyclin kinase (Fig. 1C). However, replacing that threonine by isoleucine showed no observable effect on either the nuclear localization of E1 or its efficiency in DNA replication (Lentz et al. 1993). Thus, the functional role, if any, of phosphorylation of the BPV replication proteins remains unknown.

Yeast Origin Recognition Complex

The most likely candidate for the replication initiator in yeast is the origin recognition complex (ORC) (for review, see Bell and Stillman 1992; Diffley 1992; Huberman 1992; Li and Alberts 1992; see Newlon;

Nasmyth; Stillman; all this volume). It consists of six polypeptides with molecular masses of 50, 53, 56, 62, 72, and 120 kD, respectively, that have been termed ORC-1 to ORC-6 according to the decrease in molecular weight. The ORC complex binds specifically to a bipartite site in yeast origins of replication in an ATP-dependent manner (Rao and Stillman 1995; Rowley et al. 1995). Mutations in the gene coding for ORC-2 have shown that ORC is necessary for the initiation of DNA replication and transcriptional silencing at the mating-type locus (Bell et al. 1992; Foss et al. 1993; Micklem et al. 1993; for review, see Newlon 1993; Huberman 1994; Kelly et al. 1994). ORC-6 has been shown to be important for the initiation of DNA replication (Li and Herskowitz 1993).

Evidence has been obtained by *in vivo* footprinting that ORC is bound to ARS-1 sequences in yeast cells in the same manner as it is *in vitro* (Diffley and Cocker 1992). Interestingly, ORC remains bound to the origin throughout the cell cycle. These data suggested that ORC could repress replication except in S phase, when it could be activated for replication. ORC-2 and ORC-6 contain multiple potential phosphorylation sites for cyclin-dependent kinases and other kinases (Bell et al. 1992; Li and Herskowitz 1993; Micklem et al. 1993), so that phosphorylation by cell-cycle-regulated kinases may influence ORC activity. However, mutations in the potential sites of phosphorylation by cyclin-dependent kinases have not yet revealed any loss of replication function (J. Diffley, pers. comm.). Interestingly, DBF4, the CDC7 protein kinase regulatory subunit, interacts with the yeast replication origin (Dowell et al. 1994), and activation of CDC7 by DBF4 at the G₁/S-phase transition is important for the initiation of S phase (Jackson et al. 1993; for review, see Newlon 1993; Nasmyth, this volume). Thus, the interaction of DBF4 with ORC-2 may target the CDC7 catalytic subunit to the ORC complex, enabling it to phosphorylate the ORC-2 polypeptide (for review, see Barinaga 1994). However, the functional effects of this phosphorylation have not yet been described.

Although the footprint of ARS sequences remains detectable throughout the cell cycle, there is an increase in the protected area during G₁ phase, which is indicative of prereplicative complexes binding in the vicinity of ORC (Diffley et al. 1994). The enlarged footprint may arise through the association of additional proteins with the ORC complex. There is evidence that ORC-6 interacts with CDC6 and CDC46/MCM5 proteins (Li and Herskowitz 1993), which are good candidates for components of the prereplicative complex. CDC6 of budding yeast and its counterpart Cdc18 from fission yeast are important for entry into S phase

(Kelly et al. 1993). The CDC6 gene product shows ATPase activity, but no detectable DNA unwinding activity (Zwerschke et al. 1994). CDC46 also acts at an early stage in DNA replication. The subcellular localization of CDC46 and other members of the MCM 2-3-5 family is regulated in the cell cycle (Hennessey et al. 1991; Y. Chen et al. 1992; Yan et al. 1993; for review, see Tye 1994). These proteins are nuclear only between M and S phase, consistent with the notion that they could participate in the G₁-specific enlargement of the footprint on the origin of replication, but no data are available on this point. One might expect that cell-cycle-dependent phosphorylation events would regulate the functions of the MCM proteins, but again, mutations in the potential sites of phosphorylation of MCM proteins by cyclin-dependent kinases have not affected their function (B. Tye, pers. comm.) (but see section below). It has been speculated based on their amino acid sequences that the MCM proteins may function as ATP-dependent helicases (Koonin 1993).

***Xenopus* and Mammalian Initiation Proteins**

The MCM proteins are found not only in yeast, but also in frogs, mice, and humans (for review, see Tye 1994). Their importance in control of DNA replication is underscored by their recent identification (Chong et al. 1995; Kubota et al. 1995; Madine et al. 1995) as components of a replication licensing factor that was postulated by Blow and Laskey (1988) to limit eukaryotic chromosomal DNA replication to one round per cell cycle (for review, see Coverley and Laskey 1994). Taken together, the data strongly support the notion that a complex of different MCM proteins is required for chromatin replication in the *Xenopus* in vitro system, that their presence in the nucleus before replication is necessary, and that upon replication, they are lost or become easily extractable from the nucleus. Interestingly, replication licensing factor activity is blocked by treatment of *Xenopus* extracts with protein kinase inhibitors, suggesting that phosphorylation is directly or indirectly involved in their function (for review, see Coverley and Laskey 1994; Tye 1994).

A mammalian homolog of MCM3, the P1 protein, was found associated with DNA pol- α :primase (Thömmes et al. 1992b). The nuclear localization of P1 is regulated during the cell cycle like that of its yeast and frog counterparts (Kimura et al. 1994). Nuclear P1 appears to exist in distinct subpopulations: one that is hyperphosphorylated and loosely bound in the nucleus, and another that is underphosphorylated and tightly bound in the nucleus. In early S phase, the tightly bound form of P1 was predominant, but as S phase progressed, the loosely bound P1 gradually

increased and became the major form by the end of S phase (Kimura et al. 1994). This behavior would be consistent with a role for P1 in prereplicative chromatin complexes and disassembly of these complexes after cell-cycle-dependent phosphorylation.

OTHER PROTEINS THAT ARE REQUIRED FOR DNA REPLICATION

Pol- α :Primase

Pol- α :primase is composed of four subunits with molecular masses of 180, 68, 58, and 48 kD (p180, p68, p58, and p48) (Thömmes and Hübscher 1990; Wang 1991 and this volume). All the cDNAs encoding the human, mouse, and yeast subunits have been cloned (Lucchini et al. 1987; Wong et al. 1988; Collins et al. 1993; Miyazawa et al. 1993; Stadlbauer et al. 1994). The structure and function of pol- α :primase are highly conserved in eukaryotic organisms (Wang 1991). Pol- α :primase is an essential component of the cellular replication machinery (Pizzagalli et al. 1988; Francesconi et al. 1991), where it functions both in initiation at origins of replication and in the synthesis of Okazaki fragments on the lagging strand of the replication fork (Focher et al. 1988; Prelich and Stillman 1988; Thömmes and Hübscher 1990; Wang 1991). Pol- α :primase also initiates the leading strand at the origin of SV40 replication (Tsurimoto et al. 1990). The p180 subunit harbors the DNA polymerase activity (Pizzagalli et al. 1988; Wong et al. 1988; Nasheuer et al. 1991), the p68 subunit is thought to play a role in the initiation process (Collins et al. 1993; Foiani et al. 1994), and the p58 and p48 subunits comprise the primase activity (Santocanale et al. 1993).

During activation of quiescent mammalian cells, the expression of all subunits is up-regulated. However, in actively cycling cells, gene expression is constitutive and mRNA and protein levels are only marginally enhanced prior to S phase (Wahl et al. 1988; Miyazawa et al. 1993). Thus, if pol- α :primase function is regulated during the cell cycle, it is likely to involve a posttranslational mechanism. Indeed, both the p180 and the p68 subunits are phosphorylated on serine and threonine residues in a cell-cycle-dependent manner (Wong et al. 1986). The p180 subunit is phosphorylated throughout the cell cycle but becomes hyperphosphorylated in the G₂-M transition, whereas the p68 subunit is phosphorylated beginning in S and more heavily at the G₂-M transition (Nasheuer et al. 1991).

Phosphorylation of DNA polymerase- α :primase at different points in the cell cycle has also been studied in budding yeast (Foiani et al. 1995). Both the p180 and p86 (B) subunits are phosphorylated in yeast, but only

the B subunit showed a reduced electrophoretic migration upon modification. The kinetics of phosphorylation during the cell cycle revealed that newly synthesized B subunit occurred in an underphosphorylated form during S phase and became modified during G₂. Maternal B subunit was stable and became phosphorylated in early S phase. Both populations of B subunit were dephosphorylated during exit from mitosis (Foiani et al. 1995). The observation that the B subunit is required very early in S phase for initiation, but has no effect on the enzymatic functions of yeast pol- α :primase, suggested that phosphorylation may regulate the activity of the B subunit in initiation of replication (Fig. 4) (Foiani et al. 1995).

The functional relevance of phosphorylation of pol- α :primase has been investigated in vitro. Treatment of pol- α :primase with casein kinase II in vitro phosphorylated the p180 and p58 subunits, but had no influence on either polymerase or primase activity (Podust et al. 1990). Some of the in vivo cell-cycle-dependent phosphorylation sites on the p180 and p68 subunits of mammalian pol- α :primase were shown to be phosphorylated by p34^{cdc2} kinases in vitro, implying these kinases as possible regulators. However, these kinases also had little effect on the enzymatic activities of pol- α :primase; only a slight reduction in affinity for single-stranded DNA was found (Nasheuer et al. 1991).

The functional effect of phosphorylation of pol- α :primase by cyclin-dependent kinases has recently been reexamined using purified proteins. Pol- α :primase expressed in the baculovirus system was modified on the p180 and p68 subunits by cyclin E-, A-, and B-dependent kinases (C. Voitenleitner et al., unpubl.). All of these kinases utilized pol- α :primase approximately equally well as a substrate. Phosphorylation of the enzyme did not affect its polymerase activity, in agreement with published data, although it stimulated primase activity severalfold. In contrast, the ability of pol- α :primase to initiate SV40 DNA replication in vitro in a reaction performed with purified proteins was markedly inhibited after phosphorylation by cyclin-A-dependent kinases. Treatment with cyclin-B-dependent kinase reduced initiation activity more modestly. Cyclin-E-dependent kinase caused no reduction in initiation activity; indeed, a small stimulation was observed in some experiments (C. Voitenleitner et al., unpubl.).

These results raise the question whether phosphorylation of pol- α :primase may influence its interaction with other initiation proteins. Both the p180 and the p68 subunits interact with SV40 T antigen (Dornreiter et al. 1990, 1992, 1993; Collins and Kelly 1991; Collins et al. 1993; Murakami and Hurwitz 1993), and these interactions play a sub-

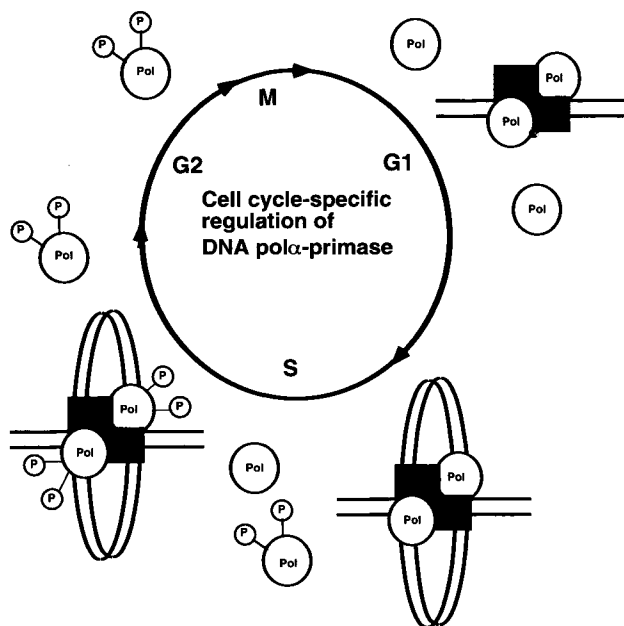


Figure 4 Model for regulation of origin-specific initiation activity of pol- α :primase by cell-cycle-dependent phosphorylation. Hypophosphorylated pol- α :primase that appears in late M or early G₁ (Foiani et al. 1995) is suggested to assemble in a prereplicative complex together with cellular initiation proteins on the cognate origin of DNA replication (Diffley et al. 1994). The complex would remain dormant until activation at the G₁/S border, an event that probably requires cyclin-E-dependent kinase directly or indirectly (Knoblich et al. 1994; Jackson et al. 1995; Sauer et al. 1995). Upon initiation, phosphorylation of pol- α :primase is postulated to inhibit its ability to initiate new rounds of replication at an origin. Perhaps phosphorylation of pol- α :primase at the origin is even involved in the transition from initiation to elongation. If cyclin A-dependent kinases, which accumulate as the cell progresses from S through M phase, were responsible for these modifications, the fraction of initiation-active pol- α :primase would be predicted to decline from S to M phase without a concomitant reduction in lagging-strand DNA replication activity. A similar mechanism may regulate the SV40 initiation activity of pol- α :primase in virus-infected cells, thereby facilitating assembly of new virus particles in late S phase and G₂.

stantial role in the initiation of SV40 replication. The ability of phosphorylated pol- α :primase to undergo these interactions has not yet been assayed directly. Intriguingly, however, only a subpopulation of mouse pol- α :primase was associated with Py T antigen, and only this population was able to stimulate viral DNA replication (Moses and

Prives 1994). It will be interesting to investigate the phosphorylation state of these different fractions of pol- α :primase.

Taken together, the results of these studies in different systems with different sets of reagents argue that cell-cycle-dependent phosphorylation of pol- α :primase regulates its function in initiation of DNA replication. Figure 4 depicts a speculative model for this regulation. Since the model makes several predictions that can be tested experimentally, it may be useful in gaining a better understanding of the role of protein phosphorylation in replication.

Replication Protein A

Replication protein A (RP-A), also known as replication factor A (RF-A) and human single-stranded DNA-binding protein (HSSB), was identified as an essential factor for SV40 DNA replication in vitro (Wobbe et al. 1987; Fairman and Stillman 1988; Wold and Kelly 1988). It is the major single-stranded DNA-binding protein in mammalian cells (Seroussi and Lavi 1993) and is highly conserved in all eukaryotes (Wold and Kelly 1988; Brill and Stillman 1989; Mitsis et al. 1993). RP-A is a heterotrimeric protein with subunits of 14, 32, and 70 kD (p14, p32, and p70) (Fairman and Stillman 1988; Wold and Kelly 1988). The cDNAs encoding all three subunits have been cloned from human (Erdile et al. 1990, 1991; Umbricht et al. 1993) and yeast (Heyer et al. 1990; Brill and Stillman 1991) and are essential for cell viability in yeast (Heyer et al. 1990; Brill and Stillman 1991; Longhese et al. 1994).

Human RP-A binds tightly to single-stranded DNA (Wobbe et al. 1987; Fairman and Stillman 1988; Wold and Kelly 1988) with low cooperativity (Kim and Wold 1995) in two distinct complexes, one occupying 8–10 nucleotides, the other 20–30 nucleotides (Blackwell and Borowiec 1994; Kim et al. 1994). Binding affinity to RNA and double-stranded DNA is much lower (Brill and Stillman 1989; Wold et al. 1989; Kim et al. 1992). RP-A has been shown to be associated with an intrinsic DNA-unwinding activity (Georgaki et al. 1992). The nucleic acid-binding properties of the homolog from *Drosophila melanogaster* (dRPA) were reported to be quite similar to those of human RP-A (Mitsis et al. 1993). However, RP-A from *Saccharomyces cerevisiae* (yRPA) was found to be quite different, binding to a site of about 90 nucleotides with high cooperativity (Alani et al. 1992). Yeast RP-A, however, cannot substitute for its human counterpart in SV40 DNA replication in vitro (Brill and Stillman 1991).

The p70 subunit of RP-A contains the intrinsic DNA-binding activity (Wold et al. 1989; Kenny et al. 1990). The p32 and p14 subunits form a stable subcomplex (Henricksen et al. 1994; Stigger et al. 1994) that is important for proper formation of the heterotrimeric complex (Henricksen et al. 1994). The biological functions of the 32-kD and 14-kD subunits are not known. However, monoclonal antibodies directed against the 70-kD, 32-kD, or 14-kD subunits block SV40 DNA replication, suggesting a role for all three subunits in this process (Erdile et al. 1990; Kenny et al. 1990; Umbricht et al. 1993).

RP-A has multiple functions in the replication process (Kenny et al. 1989). During bidirectional unwinding of the SV40 origin of replication by T antigen, it stabilizes the created single-stranded regions. Moreover, it is essential during primosome assembly (Melendy and Stillman 1993) and primer synthesis on the SV40 origin (Schneider et al. 1994), reflecting the specific protein-protein interactions that occur between RP-A, T antigen, and pol- α :primase (Dornreiter et al. 1992, 1993; Nasheuer et al. 1992; Murakami and Hurwitz 1993; K. Weissbart and E. Fanning, unpubl.). RP-A also plays a role in the elongation reaction, where it has been shown to stimulate DNA polymerases α (Kenny et al. 1989; Erdile et al. 1991; Brown et al. 1992), δ (Kenny et al. 1989; Tsurimoto and Stillman 1989; Lee et al. 1991a), and ϵ (Lee et al. 1991b). In addition, it increases the fidelity of DNA synthesis by pol- α :primase (Carty et al. 1992; Roberts et al. 1993; Suzuki et al. 1994) and stimulates copurifying helicases (Seo et al. 1991; Thömmes et al. 1992a; Georgaki et al. 1994).

Besides its essential functions in DNA replication, RP-A is also required for DNA repair and recombination *in vivo* (Longhese et al. 1994) and nucleotide excision repair of UV-damaged DNA *in vitro* (Coverley et al. 1991). It also stimulates *in vitro* strand-exchange proteins involved in homologous recombination (Heyer et al. 1990; Moore et al. 1991; Alani et al. 1992). RP-A has also been shown to interact with the acidic domains of transcription factors VP16, GAL4, and p53 (Dutta et al. 1993; He et al. 1993; Li and Botchan 1993). The significance of these interactions is still unknown. On the one hand, these factors stimulated BPV replication (Li and Botchan 1993); on the other hand, p53 inhibited SV40 replication (Dutta et al. 1993). It was found recently that yRP-A binds specifically to sequence elements that are involved in the regulation of transcription (Luche et al. 1993; Singh and Samson 1995), so that the interaction of RP-A with transcription factors could play a role in transcriptional regulation.

Several posttranslational modifications of the RP-A subunits have been observed. Both the p70 and p32 subunits are pol-ADP-ribosylated

(Eki and Hurwitz 1991) and phosphorylated (Din et al. 1990; Fang and Newport 1993). The abundance of RP-A remains constant throughout the cell cycle, but the p32 subunit is phosphorylated in a cell-cycle-specific manner on multiple serines (Din et al. 1990). In cycling cells, p32 becomes extensively phosphorylated at the G₁-S transition and remains phosphorylated until G₂-M, when dephosphorylation occurs (Din et al. 1990). The hyperphosphorylated form of p32 shows decreased mobility during gel electrophoresis and runs as a 36-kD species.

On the basis of amino acid sequence, human RP-A p32 has two potential sites for cyclin-dependent kinases, five for DNA-PK, and five for casein kinase II. There is good evidence that cyclin-dependent kinases and DNA-PK phosphorylate RP-A p32 in vitro and in vivo. In vitro, cdc2-cyclin B kinase phosphorylates p32 at Ser-23 and Ser-29, sites that are also phosphorylated in vivo (Dutta and Stillman 1992), concomitant with a modest stimulation of SV40 DNA replication and origin unwinding in G₁ extracts. The same sites were also targeted specifically within the replication initiation complex by cdk2-cyclin A kinase (Fotedar and Roberts 1991, 1992; Dutta and Stillman 1992; Elledge et al. 1992; Pan et al. 1993a). However, mutant RP-A lacking the two cyclin-dependent kinase sites in the p32 subunit supported SV40 DNA replication in vitro as well as wild-type RP-A, demonstrating that phosphorylation of RP-A by cyclin-dependent kinases is dispensable in this system (Henricksen and Wold 1994). Consistent with these results, phosphorylated and unphosphorylated RP-A were equally active in SV40 DNA replication in vitro (Pan et al. 1995).

Under the conditions of the in vitro SV40 replication system, underphosphorylated RP-A becomes hyperphosphorylated (Fotedar and Roberts 1992; Henricksen and Wold 1994). The kinase involved appears to be DNA-PK, but cyclin A-cdk2 may also play a role. RP-A p32 is predominantly phosphorylated when bound to DNA, and the kinase that phosphorylates it is itself bound to single-stranded DNA (Fotedar and Roberts 1992). The p32 subunit in RP-A from cycling embryonic *Xenopus* extracts is also phosphorylated in S phase only in nuclear-localized RP-A bound to single-stranded DNA (Fang and Newport 1993). Hyperphosphorylation of RP-A p32 by DNA-PK in a cyclin-A-activated G₁ extract was reported to require prephosphorylation by cyclin A-cdk2 (Pan et al. 1994). However, mutant RP-A lacking the two potential cyclin-dependent kinase sites in p32 still becomes hyperphosphorylated (Henricksen and Wold 1994; Lee and Kim 1995). Moreover, replication-competent extracts depleted of cyclin-dependent kinases still hyperphosphorylate DNA-bound RP-A in vitro (Dutta and Stillman

1992), whereas extracts depleted of DNA-PK do not (Brush et al. 1994).

Human RP-A is also found hyperphosphorylated in G₁ cells that have been exposed to ionizing radiation (Liu and Weaver 1993) or UV light (Carty et al. 1994). A good candidate for the kinase that phosphorylates RP-A during the damage response is the DNA-PK (Lees-Miller and Anderson 1991), a kinase that is known to be involved in DNA repair and is stimulated by double-strand breaks (for review, see Gottlieb and Jackson 1994). Purified DNA-PK phosphorylates RP-A p32 in vitro (Brush et al. 1994; Pan et al. 1994), and cells that are deficient in DNA-PK activity fail to hyperphosphorylate RP-A after irradiation (Boubnov and Weaver 1995). Hyperphosphorylated RP-A in extracts from irradiated cells appeared to be inactive in SV40 DNA replication in vitro, and additional RP-A purified from unirradiated cells relieved that block (Carty et al. 1994), suggesting that phosphorylation on the potential DNA-PK sites might activate RP-A for its tasks in repair while blocking its function in the replication process. However, other workers have found that phosphorylation of RP-A by DNA-PK did not affect its activity in SV40 DNA replication in vitro (Brush et al. 1994).

Based on the evidence from in vitro SV40 DNA replication, phosphorylation of RP-A appears to have little or no functional importance. However, it may play a role in vivo, such as correct localization to replication centers (for review, see Hozak and Cook 1994). Chromatin replication in *Xenopus* egg extracts is dependent on RP-A (Adachi and Laemmli 1994). Before being assembled into replication centers, RP-A is targeted to the prereplicative sites that form after mitosis and prior to initiation (Adachi and Laemmli 1994). This association is loose due to the absence of single-stranded DNA and is likely to be mediated by protein-protein interactions. Assembly of RP-A into prereplicative complexes does not occur in the presence of active cyclin B-cdc2 kinase, suggesting that only the underphosphorylated form of RP-A found after mitosis can be targeted to the complexes. Active cyclin A-cdk2 kinase did not facilitate or inhibit the assembly of prereplicative complexes (Adachi and Laemmli 1994). However, it colocalized with RP-A, and presumably many other replication proteins, to replication foci (Adachi and Laemmli 1992, 1994; Cardoso et al. 1993), suggesting a possible function in this process.

In summary, it seems likely that phosphorylation of RP-A regulates its activity in vivo, but the mechanism of this regulation remains poorly understood. Much of the confusion may stem from the multiple functions of RP-A in the cell, but it should be noted that the assay system used most frequently to assess the effects of phosphorylation, in vitro SV40

DNA replication, probably does not reveal all of the relevant steps in vivo regulation of DNA replication.

DNA Polymerases δ and ϵ

Polymerases δ and/or ϵ are thought to play a pivotal role in leading-strand DNA synthesis as well as lagging-strand synthesis (Burgers 1991; Nethanel et al. 1992; Podust and Hübscher 1993; see Wang, this volume). Polymerase- δ is a two-subunit complex with polypeptides of approximately 124 kD and 55 kD (Chung et al. 1991) whose activity and processivity are stimulated by complexing with PCNA (Bauer and Burgers 1988). Polymerase- ϵ consists of a catalytic subunit of 258 kD (Kesti et al. 1993) that copurifies with a smaller 55-kD polypeptide in human (Sylväoja and Linn 1989; Lee et al 1991b) and with polypeptides of 80, 34, 30, and 29 kD in yeast extracts (Hamatake et al. 1990). RF-C and PCNA also function to stimulate polymerase- ϵ (Burgers 1991; Lee et al. 1991b). Besides their involvement in DNA replication, both polymerase- δ and polymerase- ϵ are implicated in DNA repair (Z. Wang et al. 1993; Blank et al. 1994; Zeng et al. 1994b; Budd and Campbell 1995).

Polymerase- δ mRNA and protein expression is somewhat increased at the G_1 -S border, but more significantly, the protein is most actively phosphorylated during S phase (Zeng et al. 1994a). It will be important to assess the enzymatic activities of hyper- and hypophosphorylated polymerase- δ and to look for differences in its interactions with other replication proteins like PCNA (Bauer and Burgers 1988) or RP-A (Longhese et al. 1994).

Polymerase- ϵ mRNA expression and protein levels are strongly dependent on cell proliferation (Kesti et al. 1993; Tuusa et al. 1995), but their abundance fluctuated less dramatically in actively cycling cells. The phosphorylation state of polymerase- ϵ has not been investigated.

PCNA and RF-C

PCNA is a phosphoprotein and its phosphorylation state appears to vary in a cell cycle-dependent fashion (Prosperi et al. 1993, 1994). Interestingly, highly phosphorylated PCNA associated with insoluble nuclear structures primarily during S phase, whereas soluble PCNA was found in early G_1 and G_2/M in a weakly phosphorylated form. Expression of PCNA appears to be independent of the cell cycle in actively growing cells. On the other hand, its expression declines in quiescent cells and is strongly induced by mitogens (Mathews et al. 1984). It is also subject to

regulation by binding to cdk-cyclin kinases and their inhibitors (for review, see Heichman and Roberts 1994; Pines 1994).

RF-C is a multisubunit protein consisting of five polypeptides (p36.5, p37, p38, p40, and p140; also designated RFC1–5) (Lee et al. 1991b; Tsurimoto and Stillman 1991c). The mammalian and yeast genes encoding the RF-C subunits have been cloned (M. Chen et al. 1992a,b; Bunz et al. 1993; Pan et al. 1993b; Li and Burgers 1994a,b; Luckow et al. 1994; Noskov et al. 1994; Cullman et al. 1995; Hübscher et al., this volume). The p140 subunit has an ATP-binding domain and binds unspecifically to DNA. Its transcripts are most abundant in strongly proliferative tissues but are also relatively high in brain (Luckow et al. 1994). The p40 polypeptide has an ATP-binding motif and shows ATPase activity. It seems to interact with PCNA, polymerase- δ , and the p37 subunit of RF-C (Pan et al. 1993b). The steady-state levels of the mRNA fluctuate only slightly during the cell cycle (Noskov et al. 1994). The p38 subunit binds ATP, and its ATPase activity is stimulated by single-stranded DNA (Li and Burgers 1994a). The p37 subunit shows specific affinity to polymerase- ϵ and binds specifically to primer-template termini (Pan et al. 1993b). The p37 mRNA levels do not change significantly during the cell cycle (Li and Burgers 1994b). Since RNA levels of two of the subunits are constant during the cell cycle, protein modifications like phosphorylation might contribute to the regulation of RF-C function. A thorough investigation of RF-C expression and modification, however, must await the availability of suitable immunological reagents.

Topoisomerases

Topoisomerases are involved in the maintenance of chromatin and DNA structure (see Hangaard-Andersen et al., this volume). Topoisomerase I relaxes supercoiled DNA, whereas topoisomerase II catalyzes decatenation and unknotting of topologically linked DNA circles, as well as the relaxation of supercoiled DNA (Wang 1985). Both topoisomerases have been shown to be phosphorylated.

Dephosphorylation of serine/threonine residues in topoisomerase I isolated from different sources reduced or abolished its enzymatic activity (Durban et al. 1983; Kaiserman et al. 1988; Pommier et al. 1990; Tournier et al. 1992). Phosphorylation of the protein on tyrosine decreased activity as well (Tse-Dinh et al. 1984). Conversely, phosphorylation of topoisomerase I by casein kinase II and related kinases (Durban et al. 1983; Kaiserman et al. 1988) or protein kinase C (Pommier et al. 1990; Samuels and Shimizu 1992) stimulated activity.

The decatenation activity of topoisomerase II was shown to be stimulated by phosphorylation by casein kinase II, protein kinase C, cdc2-cyclin kinases, and Ca^{++} /calmodulin-dependent protein kinase (Sahyoun et al. 1986; Rottman et al. 1987; Ackerman et al. 1988; Cardenas and Gasser 1993; for review, see Poljak and Käs 1995). The major *in vivo* kinase acting on topoisomerase II in *S. cerevisiae* has been identified as casein kinase II (Cardenas et al. 1992). Topoisomerase II is hyperphosphorylated during G₂/M phase (Cardenas et al. 1992), consistent with the finding that this phosphorylation is required for activity of topoisomerase II during chromosome segregation. The stimulation of decatenation induced by phosphorylation was found to be at least partly due to an increase in the DNA-binding affinity of topoisomerase II (Dang et al. 1994) and an increased rate of ATP hydrolysis (Corbett et al. 1993).

SUMMARY AND OUTLOOK

Our understanding of the role of protein phosphorylation in regulation of DNA replication remains fragmentary. All of the proteins that recognize and activate eukaryotic origins of replication are phosphorylated, but with the exception of SV40 T antigen, there is no clear picture of how these modifications affect the function of the proteins. Similarly, the key replication enzymes in eukaryotic cells are phosphoproteins, but the functional importance of phosphorylation is established only for topoisomerase II, and in preliminary fashion for pol- α :primase. The lack of data to support a role for phosphorylation in regulation of DNA replication is not due to lack of effort. The mutational analysis of potential phosphorylation sites that was successful in investigating SV40 T antigen has now been extended to many of the cellular origin recognition proteins and several replication enzymes, including pol- α :primase and RP-A, without conspicuous success. Does this mean that these phosphorylation events are fortuitous? The cell-cycle-dependence of phosphorylation of replication proteins, the correlation between phosphorylation of MCM proteins with changes in their subcellular localization, and the importance of the cyclin-dependent kinases, other cell-cycle-dependent kinases, and the DNA-dependent protein kinase suggest that phosphorylation of most replication proteins is probably physiologically relevant. How then can one demonstrate this importance more directly? It should be noted that the current understanding of phosphorylation in control of SV40 T antigen's replication activity was not achieved solely through mutagenesis. Not only were the *in vivo* phosphorylation sites mapped, but

several overexpression systems and a wide variety of biochemical assays were available in many laboratories to probe the functions of T antigen and its mutants before and after treatment with purified kinases and phosphatases over the course of the past decade. As outlined in the introduction, such a broad approach will likely be required to understand the roles of phosphorylation in the control of DNA replication.

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