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IMPLICATION OF TRANSCRIPTION FACTOR E2F IN REGULATION OF DNA REPLICATION

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TABLE OF CONTENTS

1. ABSTRACT

The transcription factor E2F plays crucial roles in induction of S phase in mammalian cells by regulating the expression of genes that encode molecules involved in cell cycle progression. E2F exerts a repressive effect on E2F-responsive genes in G0/G1 phase by associating with the retinoblastoma tumor suppressor gene product pRb and the related protein p130. This repression is relieved by phosphorylation of the pRb family proteins by G1 cyclin (cyclin D and cyclin E) -dependent kinases, resulting in expression of E2F-responsive genes in late G1 with a peak at the G1/S boundary. One group of genes influenced by E2F encode cell cycle regulatory molecules, including members of the E2F family and cyclin E, demonstrating a loop-type regulation of activities of E2F and cyclin E-dependent kinase at this stage in the cell cycle. Another group is involved in DNA replication, including genes for molecules regulating initiation of DNA replication. Overexpression of E2F is sufficient to induce DNA synthesis in serum starved fibroblasts. In addition, overexpression of cyclin E, which is essential for entry into S phase, overcomes G1 arrest caused by inhibition of E2F activity without resuming E2F mediated transcription, suggesting the mergence of the two pathways. Thus, E2F target-gene products and cyclin E-dependent kinase activity apparently co-operate to initiate replication of DNA.

2. INTRODUCTION

The commitment to replicate cellular DNA is the most important step in cell growth control. Cell cycle progression is strictly regulated by orderly activation and inactivation of cyclin-dependent kinases (cdks), and the decision to proceed through the cell cycle is made during G1 phase, dependent on stimulation by various extracellular agents such as growth factors. After passing through a point late in G1 phase, called the restriction point, cells can proceed through the remainder of the cell cycle independent of such extracellular stimuli. Recent studies have identified the regulatory pathway critical for passage through G1 into S phase. The G1 cdks, which are activated by growth stimulation, phosphorylate pRb and related proteins p107 and p130, subsequently leading to activation of the transcription factor E2F. E2F appears to play key roles in G1/S transition through activation of a set of genes that encode cell cycle regulatory molecules as well as various genes encoding proteins important for DNA replication in S phase.

This review will summarize recent advances in understanding of the roles of E2F in regulation of cell cycle progression with a focus on regulation of DNA replication.

3. A BRIEF HISTORY

The adenovirus early gene product E1a is known to induce DNA replication in serum-starved fibroblasts, through modification of the cellular transcription program (reviewed in 1). Studies of this line identified E2F as an essential cellular transcription factor for E1a-dependent trans-activation of the adenovirus E2 gene (2). E2F is found in complexes with cellular proteins in most cell types (3, 4), and E1a has been

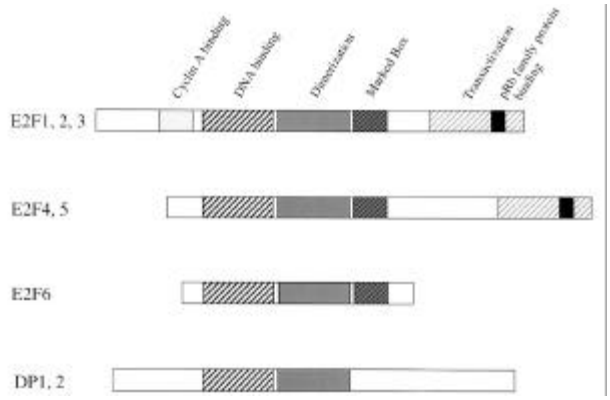

Figure 1. Schematic representation of the domain structures of E2F and DP family members.

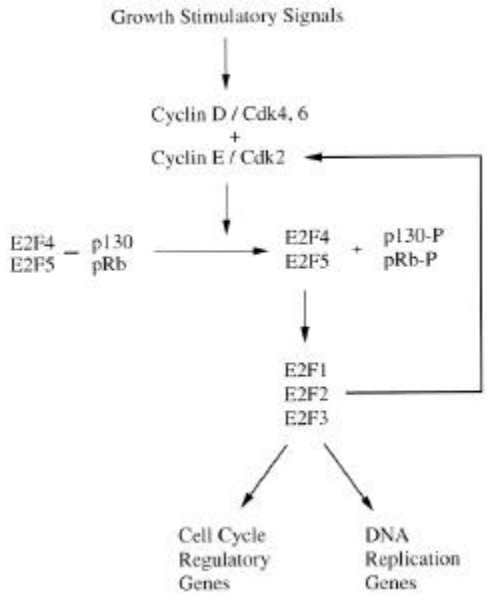

Figure 2. A cascade in activation of E2F.

shown to possess the capacity to dissociate these complexes and release free E2F molecules (4), indicating that free E2F is an active form which functions as the final mediator of E1a actions (5, 6). E2F was found to bind to sites in promoter regions of cellular genes encoding proteins involved in cell cycle and DNA synthesis (7-9), implicating E2F in cell cycle control, including DNA replication. It was further suggested that DNA replication induced by E1a is mediated by E2F through activation of cellular genes regulating the cell cycle and DNA synthesis.

Detection of the retinoblastoma tumor suppressor gene product (pRb) as an E1a binding protein (10) suggested that pRb might be one of the cellular proteins complexing with E2F. Indeed, physical interaction between pRb and E2F has been identified (11-14), and this correlates with the ability of pRb to function as a growth suppressor (15, 16), indicating that binding to and consequent inactivation of E2F is one of its important downstream effects. In addition to adenovirus E1a, large T antigens of SV40 and polyoma viruses, and the E7 gene product of human papilloma virus also interact with pRb (17-19) and dissociate E2F-pRb complexes (20). Thus activation of E2F appears to be a common mechanism by which these DNA viruses induce DNA replication in host cells to facilitate viral genome replication. In this context, it is noteworthy that cellular DNA replication in serum-starved fibroblasts is induced by E2F1, a component of E2F, as efficiently as by E1a (21). The results underscore the critical roles of E2F in regulation of DNA replication in mammalian cells.

4. MOLECULAR BASIS OF E2F ACTIVITY

E2F is now known to comprise heterodimers of members of the E2F and DP families of proteins (figure 1) (reviewed in 22-24). To date, six members of the E2F family (E2F1 through E2F6) and two members of the DP family (DP1 and DP2) have been identified (25-41). E2F heterodimers are formed via interaction of the dimerization domains of two family members, the consequence being that complexes are able to bind to E2F binding sites (consensus sequence: TTT $^{C}_{G}$ $^{C}_{G}$ CGC) in promoter regions through DNA binding domains of both family proteins. Carboxy terminal transactivation domains of E2F1 through E2F5 are necessary for transcriptional activation, but are lacking in DP family members and E2F6. Transcriptional activation is therefore absolutely dependent on the participation of a member of the E2F family, and thus heterodimers between E2F and DP family members are referred according to the member of E2F family.

5. REGULATION OF E2F-MEDIATED TRANSCRIPTION

Transcriptional activity of E2F is tightly regulated throughout the cell cycle. In quiescent cells (G0 phase), it is suppressed by binding to pRb family proteins. Upon growth stimulation, E2F activity is dramatically induced in late G1 and peaks at the G1/S boundary. E2F activity is regulated by multiple mechanisms during the cell cycle.

5.1. Binding by pRb family proteins

Binding of E2F to pRb family proteins is the primary regulatory mechanism for E2F activity (reviewed in 24, 42-44). In quiescent cells, E2F is present in an inactive form bound to pRb and p130 (45, 46). Phosphorylation of the pRb family proteins by G1 cdks, which are activated by growth stimulation, inhibits binding of those proteins to E2F, allowing accumulation of free E2F (figure 2) (13, 47). pRb family proteins contain multiple consensus sites for phosphorylation by G1 cdks. Hypophosphorylated forms of the pRb family proteins have high affinity for E2F while hyperphosphorylated forms have very low affinity. The cdks associated with G1 cyclins, cyclin D and cyclin E, appear to co-operate in phosphorylation of pRb (48-50).

The pRb family proteins bind to E2F1 through E2F5 through the pRb binding domain embedded in the transactivation domain of E2F family proteins, thus abolishing

transactivating ability of E2F apparently by physically blocking the activation domain. Heterodimerization between E2F and DP family members is required for efficient binding to pRb family proteins (51, 52). Since E2F6 does not have the transactivation domain it cannot bind to pRb family proteins (39, 41, 53). Instead it behaves as a repressor of E2F mediated transcription. How E2F6 is regulated during the cell cycle is not yet understood.

There are differences in affinity of pRb family proteins for E2F members (44, 54, 55). It is generally accepted that pRb preferentially binds E2F1 through E2F3, whereas p107 and p130 preferentially bind E2F4 and E2F5. However, pRb can also bind E2F4, depending on the cellular circumstances (54, 55).

5.2. Release from repression: A mechanism of E2F-mediated activation

Binding to pRb family proteins apparently not only abrogates the transactivating ability of E2Fs but also actively suppresses the activity of promoters containing E2F sites (56). Promoter activity in G0/G1 phase was found to be increased for B-myb, E2F1, E2F2, cyclin E, HsOrc1, HsCdc6, HsMCM5 and CDC2 promoters by mutations which abolished binding of E2F (57-67). The E2F sites in these promoters act as repressor elements during G0/G1 and mutations release the repression, implying that they are normally under the control of pRb family proteins. Indeed, pRb has been demonstrated to repress transcription when fused to the DNA binding domain of a heterologous transcription factor, depending on the presence of binding sites for the factor in the promoter (68-71). In addition, expression of an E2F mutant containing only the DNA binding domain blocks repression of E2F site caused by expression of $p16^{INK4a}$, an inhibitor of cyclin D dependent kinases (72).

Considering that the major E2F binding activity in the quiescent state is due to E2F4 and E2F5 bound to p130, this pRb family member is considered to be the predominant repressor under this condition (45, 46, 55). As the cell cycle proceeds through G1, E2F-p130 complexes disappear, presumably due to phosphorylation of p130 by G1 cdks and degradation via the ubiquitin-proteasome pathway (46, 73, 74). Concomitantly, free E2F begins to accumulate. Conversely, expression of pRb and p107 increases as cells reach the G1/S boundary, presumably, at least in part, through activation by E2F (75, 76). At the G1/S boundary, E2F exists partly in the free form and the remainder is complexed with p107 and pRb, depending on the degree of phosphorylation of these proteins. This presumably is one determinant of E2F activity during the cell cycle.

Several possibilities have been proposed for active transcriptional repression mediated by pRb family proteins. Their binding to promoters through E2F may inactivate surrounding transcription factors by blocking their interaction with the basal transcription complex (71). The pRb family proteins, when bound to DNA through E2F, are thought to recruit other proteins to repress transcription. hBrm and BRG1, components of mammalian SWI/SNF complexes, which are implicated in transcriptional regulation through remodeling of chromatin structure, co-operate with pRb family proteins in growth suppression (77, 78). In addition, hBrm co-operates with pRb to repress E2F1-mediated transactivation (79). Another molecule physically associated with pRb (80-82) and also with p107 and p130 (83) is a histone deacetylase, HDAC1, which is thought to repress transcription from promoters with E2F sites through changes in chromatin structure resulting from deacetylation of histones.

5.3. Induction of new E2Fs

In addition to the control exerted on E2F by pRb family proteins, E2F activity is influenced by expression of E2F family members themselves. In contrast to E2F4 and E2F5 which are expressed throughout the cell cycle (32, 35, 55), expression of E2F1 and E2F2 is dramatically induced, mainly at the level of transcription, as cells progress from G0/G1 to the S phase (22, 26, 59, 60, 65, 84). E2F1 and E2F2 promoters have been shown to possess E2F binding sites that function as pivotal elements in their regulation (59, 60, 61, 65). Growth stimulation induces phosphorylation of p130 and pRb by G1 cdks, releasing free E2F4 and E2F5 and allowing accumulation of free E2Fs, respectively (46, 74). E2F4 and E2F5, in turn, activate E2F1 and E2F2 expression, which causes further activation via positive feedback (figure 2). Expression of E2F3 also increases during the G1/S transition (22, 55). The E2F3 promoter seems to be regulated in a manner similar to E2F1 and E2F2 promoters (85). In addition, E2F3 protein accumulation is also affected, at least in part, by an increase in protein stability (86).

The fact that the E2F2 promoter contains potential c-Myc binding sites suggests an involvement of c-Myc in its expression (65). In addition, it is known that c-Myc can collaborate with activated Ras to induce E2F1 expression and generate cyclin E-dependent kinase activity in serum-starved REF52 cells (87). Thus, one of the roles of c-Myc in promotion of cell growth is apparently activation of E2F.

5.4. Subcellular localization

E2F4 lacks a nuclear localization signal (NLS) and is distributed in both the nucleus and cytoplasm of quiescent cells and mostly in the cytoplasm once cells reach S phase, in contrast to E2F1 through E2F3 which contain an NLS and localize primarily in the nucleus (88-90). Of the E2F family, E2F4 protein is predominant throughout the cell cycle (32, 54, 55): E2F1 through E2F3 are less abundant, even at the G1/S boundary. However, because of the relocalization of E2F4 in the cytoplasm, the amount in the nucleus is limited and a good proportion of nuclear E2F binding activity is comprised by E2F1 and E2F3 at the G1/S boundary *(see for example* 91). As the cytoplasmic relocation of E2F4 coincides with the timing of activation of E2F-responsive genes, this may be a mechanism preventing repression of target genes by E2F4-containing repressor complexes.

5.5. Down-regulation of E2F activity

As cells leave S phase, the expression of E2F target genes such as PCNA and cyclin E decreases *(see for example* 91) and ectopic expression of constitutively active forms of E2F1 or DP1 causes accumulation of cells in S phase (92). These results imply that E2F activity needs to be down-regulated for cells to exit S phase. Since hyperphosphorylation of pRb continues to the end of M phase, its binding is

Table 1. Genes with E2F sites

Cell cycle regulators
- Cyclin D1, D2, E, A
- CDC2
- E2F1, 2
- RB, p107
- c-myc, N-myc, c-myb, B-myb

Enzymatic machinery for DNA replication
- dihydrofolate reductase (DHFR)
- DNA polymerase alpha
- proliferating cell nuclear antigen (PCNA)
- thymidine kinase
- thymidylate synthetase
- ribonucleotide reductase

Regulatory machinery for DNA replication
- Orc1
- Cdc6
- MCMs
- CDC45

apparently not responsible, and there appear to be other mechanisms for down-regulation of E2F activity.

E2F1 through E2F3 have cyclin binding domains which mediate stable association with cyclin A/cdk2 (93-95) and phosphorylation of E2F1 and DP1 by cyclin A/cdk2 inhibits DNA binding activity of the E2F1/DP1 heterodimer (93- 97). Thus it is conceivable that such phosphorylation down-regulates E2F activity late in S phase. Free E2F is susceptible to degradation via the ubiquitin-proteasome pathway and binding by pRb family proteins protects against this (98-100). Whether ubiquitin-directed breakdown of free E2F also contributes to its down-regulation in S phase remains to be elucidated.

5.6. Expression of E2F-regulated genes in cycling cells

Restimulation of fibroblasts synchronized at G0 phase by serum starvation has been used in many studies to address the question of how expression of E2F target genes is regulated during re-entry into the cell cycle (i.e. from G0 through G1 and into the S phase). Although the degree of change in normally cycling cells is not as dramatic as that observed during re-entry into the cell cycle, mRNA levels of certain E2F target genes, such as cyclin E and Cdc6, fluctuate with a peak at the G1/S boundary, indicating induction of E2F activity at that point.

5.7. Contribution of other transcription factors

E2F regulated genes contain binding sites for other transcription factors, such as Sp1 and C/EBP, in addition to E2F binding sites. Mutation or deletion of these elements reduces the promoter activities of these genes (*see for example* 59, 60), suggesting possible interaction of other transcription factors with E2F. Indeed, physical interaction between Sp1 and E2F has been reported (101, 102). These results suggest that E2F is not solely important for regulation of the genes, but other regulatory factors are involved in this process.

6. E2F TARGET GENES

Identification of the E2F binding consensus site prompted the search for E2F-regulated genes, most so far identified being related to cell cycle progression (table 1). These genes may be divided into three groups: one encoding molecules involved in cell cycle control, a second encoding components of the enzymatic machinery for DNA replication and the third encoding DNA replication regulatory molecules.

6.1. Cell cycle regulatory machinery

Cyclin E mRNA and protein levels fluctuate during the cell cycle with a peak at the G1/S boundary (91, 103). Since the stability of cyclin E mRNA does not significantly change during the cell cycle (104) and activity of the cyclin E promoter after growth stimulation parallels the amount of mRNA (62, 63, 105), the critical regulation occurs at the level of transcription. Human and murine cyclin E promoters have been shown to contain multiple E2F sites which are responsible for cell cycle-regulated expression (62, 63, 105), pointing to E2F as an upstream regulator.

Considering that cyclin E-dependent kinase is involved in phosphorylation of pRb, this suggests a positive feedback loop at the G1/S boundary (figure 2). This loop-regulation of E2F and cyclin E-dependent kinase activity is thought to generate a rapid rise in both activities as cells approach the G1/S boundary (106). Cyclin D dependent kinases are also involved in phosphorylation of pRb. Although promoters of the cyclin D1 and D2 genes have been shown to contain typical E2F recognition sites (107, 108), the timing of expression of these genes is much earlier than that of activation of E2F and the exact role of E2F in their expression during the cell cycle remains to be elucidated. It appears that expression of D-type cyclins is largely dependent on mitogenic stimulation (106). Taken together, pRb inactivation may shift from being mitogen-dependent (cyclin D-driven) to mitogen-independent (cyclin E-driven), at the G1/S boundary, leading to an irreversible commitment to enter S phase (106). This would imply that E2F activity is intrinsically involved in G1 to S phase progression by regulating expression of cyclin E and consequently cyclin E-dependent kinase activity.

Expression of cyclin A is induced from the G1/S boundary and peaks in S phase. Variant E2F sites in the cyclin A promoter are responsible for expression of the cyclin A gene (109), and cyclin A is essential for entry into S phase (110, 111). Because of the late appearance of cyclin A as compared to cyclin E, it is thought to play a role(s) subsequent to initiation of DNA replication. The CDC2 gene product, involved in the G2/M transition by forming complexes with cyclin B and cyclin A, is induced from the G1/S boundary and this is mediated by E2F sites in the promoter (57). The results indicate that expression of cell cycle regulators which function after the G1/S transition is also under the control of E2F, suggesting an additional role in cell cycle progression at later stages.

c-Myc is a transcription factor intimately involved in cell cycle control (reviewed in 112). This is exemplified by the induction of S phase found on activation of c-Myc in quiescent fibroblasts in the absence of growth factors (113). As the timing of c-myc expression is earlier than activation of E2F, exact roles of E2F in control of c-myc expression during the cell cycle have yet to be established. However, the E2F sites in the c-myc P2 promoter may be responsible for transactivation of c-myc by E1a (8, 9).

6.2. Enzymatic machinery for DNA replication

The enzymes responsible for DNA replication include dihydrofolate reductase (DHFR), DNA polymerase alpha, thymidine kinase, thymidylate synthetase, and ribonucleotide reductase (reviewed in 22, 114). Expression of the all genes for these is induced by growth stimulation with a peak at the G1/S boundary and E2F sites in the promoters of these genes have been shown to act as major regulatory elements during the cell cycle.

6.3. Regulatory machinery for DNA replication

Recent progress in the area of yeast genetics has identified several genes which are involved in regulation of the initiation of DNA replication (reviewed in 115, 116). These include those for origin recognition complex (ORC) -1 through -6, CDC6, maintenance of minichromosome (MCM) -2 through -7, CDC7 and DBF4 kinase complex, and CDC45. ORC marks the place where DNA replication takes place by binding to replication origins and remaining there throughout the cell cycle, suggesting that regulation of initiation of DNA replication is mediated by other interacting factors. The MCM family proteins are thought to be components of a putative licensing factor. CDC6 has been shown to function in determining rate of firing of origins and in loading of MCMs onto the origins (117, 118). The CDC7 and DBF4 kinase complex appears to be required to trigger initiation of DNA replication and a *Xenopus* homolog of CDC45 has been shown to play a role in loading DNA polymerase alpha onto chromatin (119). Although not all of these genes are cell cycle-regulated in yeast, this is clearly the case for CDC6 and DBF4, with expression peaking at the G1/S boundary. It is interesting to note that the CDC6 and DBF4 genes contain a MluI cell cycle box (MCB) in their regulatory regions, binding the MCB binding factor (MBF), a functional homolog of E2F (120, 121).

Although discrete origin sequences have not been identified in mammalian cells, the existence of human and other mammalian homologs of ORC has suggested that regulatory mechanisms of initiation of DNA replication are well conserved among higher eukaryotes (122). Expression of the human homolog of the ORC1 gene, HsOrc1, is dramatically induced upon growth stimulation with a peak at the G1/S boundary from scarcely detectable levels in serum-starved primary human fibroblasts. The expression kinetics are linked to two overlapping E2F sites in the promoter, which are critical for the cell cycle-regulation (64). In addition, overexpression of E2F1 induced endogenous Orc1 gene expression in rat fibroblast REF52 cells. The HsOrc2 gene differs from the HsOrc1 gene in that expression remains relatively constant irrespective of the point in the cell cycle (64). The same is true for the recently identified HsORC4 and HsORC5 genes (123-125). Little is known about the promoters of these genes. Expression of the HsOrc1 gene therefore seems to be the most dramatically cell cycle-dependent of the human ORC homologs identified to date, suggesting that its regulation has a role in initiation of DNA replication (64).

Expression of a human homolog of CDC6, human $p62^{cdc6}$ (HsCdc6), is also growth-regulated: both mRNA and protein levels fluctuate during the cell cycle with peaks at the G1/S boundary (91, 126, 127). The HsCdc6 gene promoter contains two E2F binding sites (126), primarily responsible for its cell cycle-regulated expression (66, 127, 128). The functional significance of this was demonstrated by microinjection of antibodies against HsCdc6 protein, which resulted in prevention of S phase progression (127, 128). In this regard, it is interesting to note that in a mammalian cell-free DNA replication system, addition of recombinant *Xenopus* Cdc6 protein to permeabilized G1 nuclei of NIH 3T3 cells caused premature entry into the S phase (129). Interestingly, in this system, binding of MCM proteins to chromatin follows the kinetics of Cdc6 accumulation, and their maxima coincided with replication competence.

Six mammalian homologs of the MCM family members (MCM2 through MCM7) have been identified (reviewed in 130), all being dramatically induced by growth stimulation of serum-starved human fibroblast KD cells and rat fibroblast REF52 cells, with peaks at the G1/S boundary (91, 131). Cell cycle-dependent fluctuation has been demonstrated at the mRNA level with MCM2, 4, 5 and 6, and at the protein level with Mcm6 in human epithelial HeLa and REF52 cells (91, 132). Human MCM5, MCM6 and MCM7, and murine MCM3 have been shown to contain E2F sites in their promoters, and at least for human MCM5 and MCM6 these E2F sites are primarily responsible for the cell cycle-regulated expression (67, 131-134). Considering the fact that overexpression of E2F causes induction of all endogenous MCM family mRNAs in REF52 cells (67, 91), it is likely that there is a common regulation by E2F.

In addition to the genes already mentioned, mammalian homologs of CDC7 and CDC45 genes have also been recently identified and shown to be expressed in a cell growth-regulated manner with a peak at the G1/S boundary (135-138). While the protein level of HsCdc45 is constant throughout the cell cycle in cycling HeLa cells (138), promoter analyses have demonstrated that growth-regulated expression of the gene is essentially mediated by E2F sites in the promoter region (unpublished data).

7. ROLES OF E2F IN INITIATION OF DNA REPLICATION

The notion that E2F activity is rate-limiting for entry into S phase has emerged from accumulating evidence provided by various studies of impairment of E2F function in dominant-negative mutants (139, 140), as well as use of an RNA ligand for E2F binding (141), a transrepression domain of pRb fused to the E2F1 DNA binding domain (70) and antibodies against E2F3 (91). The results suggest that genes regulated by E2F are required for progression through G1 phase and entry into S phase.

Which E2F target genes are necessary for entry into S phase? Because of the nature of the gene products, replication regulatory genes appear to be of most importance. In contrast, genes encoding enzymatic machinery, which are relatively stable and abundant, may not be rate-limiting.

7.1. Induction of DNA replication by overexpression of E2F or cyclin E

Consistent with previous observations, overexpression of E2F1 alone was found to be sufficient to

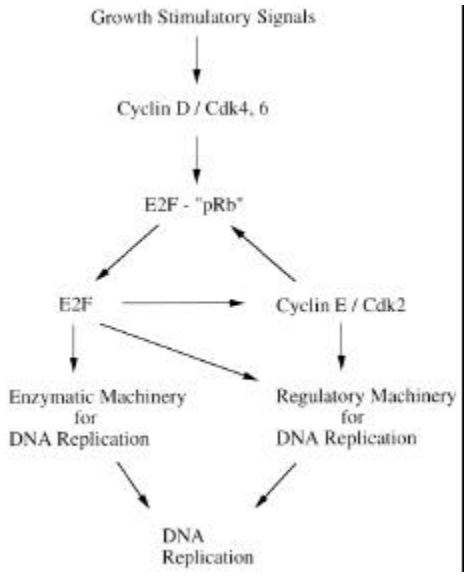

Figure 3. Collaboration of E2F and cyclin E/cdk2 in induction of DNA replication.

induce cellular DNA synthesis in serum-starved fibroblasts (21, 142-144). Similar results have been reported for E2F2, E2F3, E2F4 and E2F5 (145, 146). In addition, overexpression of E2F1 has been shown to override arrest by various negative growth signals including $p16^{INK4a}$, gamma-irradiation, TGF-beta treatment and a dominant-negative cdk2, and drive cells into S phase (145, 147-150).

In addition to the homology to yeast counterparts, circumstantial evidence supports roles for the mammalian homologs of regulatory molecules in initiation of DNA replication. It is obvious that E2F induces expression of genes encoding molecules crucial for initiation of DNA replication, at least some of which are rate-limiting. Due to properties of cell cycle-regulated expression and their homology with CDC6 and ORC1, HsCdc6 and HsOrc1 have been thought to be critical for initiation of DNA replication. Indeed, enhanced expression of the *Drosophila* homolog of ORC1 has been shown to facilitate entry into S phase *in vivo* (151). However, overexpression of each of these molecules alone did not induce S phase in serum-starved REF52 cells (66).

It is possible that induction of a single E2F target gene is not sufficient for induction of DNA replication in serum-starved fibroblasts and that multiple targets co-operate to induce DNA synthesis. Alternatively, regulatory molecules may require post-transcriptional modification by cell cycle regulators such as cdks. In this regard, it is noteworthy that overexpression of cyclin E is able to accelerate G1/S progression and to shorten G1 phase in cycling HeLa cells and serum-starved Rat-1 fibroblasts (152-155).

Cyclin E-dependent kinase is involved in phosphorylation of pRb and consequently activation of E2F. While administration of anti-cyclin E antibodies into pRb-deficient cells blocked induction of DNA synthesis (155), this was not the case with anti-cyclin D antibodies or $p16^{INK4a}$, an inhibitor of cyclin D-dependent kinases (155-157). Thus cyclin E-dependent kinase activity is essential for S phase entry, distinct from phosphorylation of pRb. Indeed, overexpression of cyclin E has been shown to bypass G1 arrest imposed by $p16^{INK4a}$, phosphorylation-deficient mutant pRb or dominant-negative mutant DP1, all of which inhibit E2F function, and to induce S phase without significant phosphorylation of pRb and E2F-mediated transactivation (158-160). In addition, studies in *Drosophila* demonstrated that cyclin E function is indispensable for S phase entry. Activation of dE2F, a Drosophila homolog of E2F, without cyclin E, is not sufficient for S phase entry and ectopic expression of cyclin E alone in early G1 phase bypassed dE2F and induced S phase (161), indicating facilitation of S phase entry independent of E2F activation.

7.2. Co-operation of E2F and cyclin E-dependent kinase in induction of DNA replication

The requirement of cyclin E dependent kinase activity for S phase entry, without activation of E2F, is in apparent contradiction to the observation that overexpression of E2F1 can drive cells into S phase without significant activation of cyclin E/cdk2 in gamma-irradiated REF52 cells (148). However, this discrepancy could be explained if cyclin E-dependent phosphorylation events and action of E2F target gene products converge on ORC, regulating the initiation of DNA replication (23)(figure 3). Overexpression of E2F target gene products could thus bypass the necessity for phosphorylation whereas enhanced phosphorylation might lower the required protein levels of E2F target gene products.

Conversely, in normal cell cycle progression, it is reasonable to speculate that E2F target gene products and cyclin E-dependent kinase activity co-operate to induce DNA replication, considering that both activities increase simultaneously at the G1/S boundary in a cross-regulated manner. Indeed, E2F and cyclin E have been shown to collaborate to induce S phase in serum-starved REF52 cells (23, 162). In addition, coinjection of human E2F1 and cyclin E into immature *Xenopus* oocytes caused DNA replication, whereas neither one was effective alone (163).

Is there a common target for E2F and cyclin E-dependent kinase? Combined expression of HsCdc6 and cyclin E synergistically induces DNA replication in serum-starved REF52 cells (164), despite the inefficiency of HsCdc6 overexpression alone (66), and increases the population of asynchronously growing human osteosarcoma U2-OS cells in S phase (128), These results imply co-operation of HsCdc6 and cyclin E-dependent kinase in induction of DNA replication. It is not known at present whether cyclin E-dependent kinase directly phosphorylates Cdc6 and modifies its functions.

At present, less than ten proteins are known to be substrates for cyclin E-dependent kinase. Of these, other than the pRb family members and a cdk inhibitor $p27^{Kip1}$ (165), NPAT (nuclear protein mapped to the AT locus) appears to have the most major involvement in S phase progression. It associates with and is phosphorylated by cyclin E/cdk2 (166), and its overexpression accelerates S phase entry, an effect enhanced by coexpression of cyclin E/cdk2. NPAT protein levels peak at the G1/S boundary (166) and the NPAT

promoter has been reported to contain E2F-like sequences (167). Thus it is likely that NPAT is affected by both E2F and cyclin E/cdk2. However, whether it directly contributes to regulation of initiation of DNA replication is not known at present. In this context, the recently reported involvement of NPAT in histone gene transcription is of interest (168).

8. PERSPECTIVES

Molecular mechanisms underlying the initiation of DNA replication in mammalian cells are still largely unknown. It remains difficult to identify discrete replication origins in the mammalian genome and thus the approach taken has been to focus on mammalian homologs of yeast genes which interact with origins and regulate DNA replication. The fact that homologs have been found for a large variety of genes, strongly suggests conservation of basic regulatory mechanisms of DNA replication among yeasts and mammals. Expression of most of these mammalian homologs has been shown to be regulated by E2F and it is likely that this is also the case for other as-yet-unidentified molecules. In fact, E2F could be a tool for identification of other participating players.

Cyclin E-dependent kinase activity appears to be critical for initiation of DNA replication. Nevertheless, no essential substrate has so far been identified. To obtain a better understanding, it is important that attention be concentrated on such replication-associated cyclin E-dependent kinase targets. It is likely that this kinase functions through phosphorylation of molecules whose expression is regulated by E2F. To elucidate the molecular mechanisms of regulation of DNA replication initiation in mammalian cells, it is crucial that the roles of these E2F target gene products be established. It is also important to determine how cdk activities co-operate with these gene products and orchestrate the initiation and progression of DNA replication.

9. ACKNOWLEDGMENTS

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