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Activity of the Retinoblastoma Family Proteins, pRB, p107, and p130, during Cellular Proliferation and Differentiation

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ABSTRACT: Genetic evidence from retinoblastoma patients and experiments describing the mechanism of cellular transformation by the DNA tumor viruses have defined a central role for the retinoblastoma protein (pRB) family of tumor suppressors in the normal regulation of the eukaryotic cell cycle. These proteins, pRB, p107, and p130, act in a cell cycle-dependent manner to regulate the activity of a number of important cellular transcription factors, such as the E2F-family, which in turn regulate expression of genes whose products are important for cell cycle progression. In addition, inhibition of E2F activity by the pRB family proteins is required for cell cycle exit after terminal differentiation or nutrient depletion. The loss of functional pRB, due to mutation of both *RB1* alleles, results in deregulated E2F activity and a predisposition to specific malignancies. Similarly, inactivation of the pRB family by the transforming proteins of the DNA tumor viruses overcomes cellular quiescence and prevents terminal differentiation by blocking the interaction of pRB, p107, and p130 with the E2F proteins, leading to cell cycle progression and, ultimately, cellular transformation. Together these two lines of evidence implicate the pRB family of negative cell cycle regulators and the E2F family of transcription factors as central components in the cell cycle machinery.

KEY WORDS: cell cycle, E2F, transcription tumor suppressor.

I. OVERVIEW

Progression through the eukaryotic cell division cycle is regulated by the synergistic activities of both positive and negative regulatory factors. The activity of these factors dictate exit from or entry into the cell cycle and control progression during G_1 and

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G_2 toward the two major events of the eukaryotic cell cycle, DNA synthesis (S phase) and mitosis (M), respectively (for reviews see Refs. 58, 90, 114, 131, 134). For the G_2 to M transition, it is well established that a mechanism, universal in all eukaryotes, operates to control entry into and progression through mitosis (for review see Ref. 133). The G_1 to S transition, while having similarities to control of entry into M, appears to have considerably greater complexity, being governed by an apparently more diverse set of signals that ultimately lead to the duplication of the genome during S phase. Genetic evidence from retinoblastoma patients and the profound effects of the DNA tumor viruses on cellular proliferation and differentiation clearly indicate that both the E2F and pRB family proteins play a fundamental role in cell cycle control. Disruption of the normal activities of these families results in the dysregulation of proliferation that is one of the hallmarks of transformed cells. The primary focus of this review is discussion of the role of the pRB family proteins in controlling the activity of the E2F family of transcription factors.

II. THE EUKARYOTIC CELL DIVISION CYCLE

Progression through or exit from the eukaryotic cell division cycle is dependent on the synergistic interactions of many positive and negative cell cycle regulators (for reviews see Refs. 70, 153, 154). As discussed in more detail below, the pRB family proteins represent an important class of negative cell cycle regulatory proteins. The best studied activity of the pRB family members is their association with and repression of the activity of the E2F family of transcription factors. The presence in the pRB family proteins of many Ser or Thr residues contained within consensus sequences recognized by the class of protein kinases known as the cyclin-dependent kinases (cdk's, for review see Ref. 123) suggested that the cell cycle-dependent activities of the cdk's were fundamental to regulation of pRB and E2F activity.

Work performed on a variety of eukaryotes, particularly the budding and fission yeast, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, respectively, demonstrated that a fundamental mechanism controlling cell cycle progression is inherent in all eukaryotes (for review see Ref. 133). At the heart of this machinery are the cyclins, whose expression levels oscillate in a cell cycle-dependent manner (see Figure 1; Refs. 30, 166, 168). The oscillations in cyclin levels result in cell cycle-dependent changes in the kinase activities of the cyclin-dependent kinases (cdk's) associated with the cyclins.[162] A relatively restricted set of factors regulates the progression from the G_2 phase of the cell cycle into mitosis (M).[133,143] Specifically, kinases homologous to the fission yeast kinase, cdc2, in conjunction with the mitotic cyclin, cyclin B, determine the G_2-M transition.

As predicted, a similar mechanism controls the G_1 to S transition in eukaryotes, although it has become apparent that a considerably greater number of homologous factors are involved in this latter transition. For example, *S. cerevisiae* employs three different cyclins, Cln1, Cln2, and Cln3, to control passage through "Start", the point in G_1 at which yeast cells are committed to undergo completion of one cell cycle.[49,171,183] Similarly, mammalian cells employ a number of "G_1 cyclins" whose expression and associated kinase activity determines progression into S phase.[85,98,110,124,185] Three cyclins, D1, D2, and D3, are expressed relatively early following mitosis or when cells enter the cell cycle from a quiescent state.

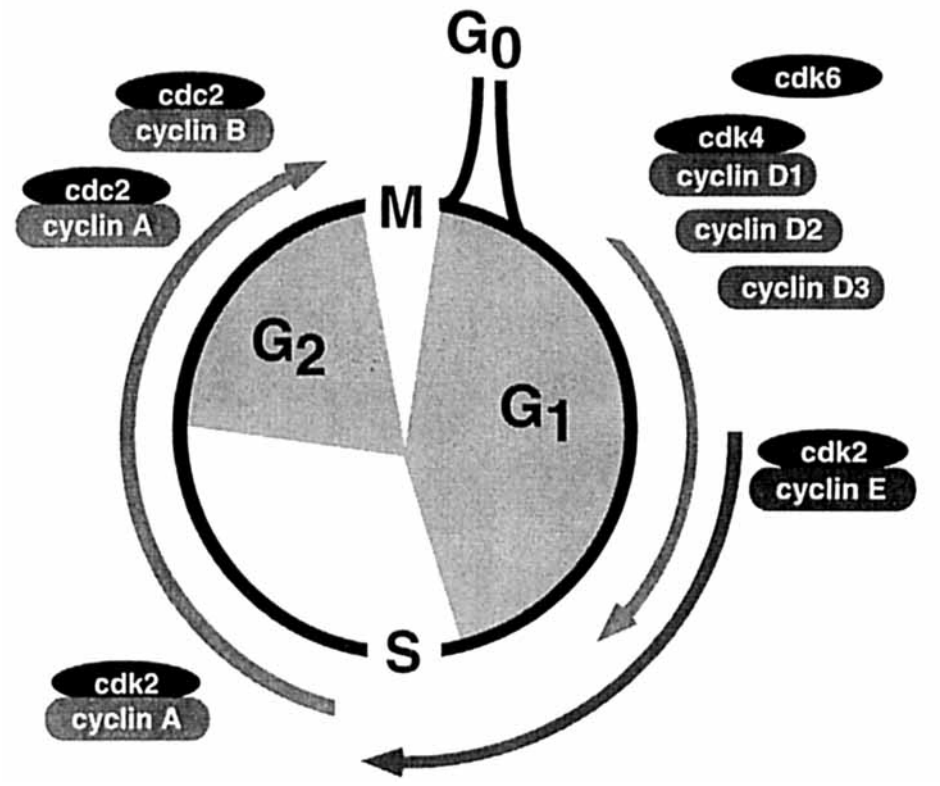


FIGURE 1. Cyclin expression during the cell cycle. The mammalian cell cycle involves the sequential expression of various cyclins in order to progress through S phase as well as to traverse the G_2/M and G_1/S boundaries. Cyclins D1, D2, and D3 associate with the cyclin-dependent kinases cdk4 and cdk6 early in G_1 following mitosis, while cyclin E, whose peak of expression is observed at the G_1/S boundary, complexes cdk2. These G_1 cyclins activate kinase activity allowing progression through G_1 and into S phase. Cyclin A, whose expression begins early in S phase, initially binds cdk2 and then cdc2 as cells approach the G_2/M boundary. Cyclin B binds cdc2 late in G_2 and both A/cdc2 and B/cdc2 complexes are required for the G_2/M transition.

Later, cyclin E is expressed with the peak of its expression observed at the G_1-S boundary, followed by cyclin A and cyclin B expression. The D-type cyclins show preferential association with cdk4 or cdk6,[2,109] while cyclin E associates with cdk2.[24,85,86] Cyclin A complexes cdk2 during S,[45,136,170,182] although it also associates with the mammalian *cdc2* homologue, p34^{cdc2}.

As Figure 1 depicts, the cell cycle can be viewed as "the cyclin cell cycle" because the expression of the cyclins marks various stages of the cell cycle and are required for progression through specific restriction points. Thus, it has been observed that inhibition of cyclin E expression or its associated kinase activity, for example, halts cells late in G_1.[81,135] Similarly, expression of cyclin A is rate limiting for progression through S phase.[144] Conversely, overexpression of these cyclins accelerates the G_1 to S transition. As might be expected, but beyond the scope of this review, inhibitors of the cyclin/cdk complexes also play a role in the cell cycle-dependent kinase activity of these com-

plexes (for reviews see Refs. 69, 70, 154, 155).

The best studied function of the cyclin/cdk complexes is to phosphorylate a variety of nuclear factors. Phosphorylation of these substrates alters their activity, facilitating progression into or through the cell cycle. The cyclin/cdk complexes also form stable tertiary complexes with some of these same nuclear factors providing an additional level of cell cycle regulation. As discussed below, two important targets of cyclin/cdk activity are the pRB family of negative cell cycle regulators and the E2F family of transcription factors.

III. ISOLATION, STRUCTURE AND EXPRESSION OF THE pRB FAMILY PROTEINS

Two convergent areas of research have been fundamental in elucidating the identity and function of pRB and E2F families of cell cycle regulatory factors. These two areas, cloning of the retinoblastoma susceptibility gene and elucidating the mechanism of cellular transformation by the DNA tumor viruses, together have led to an understanding of the role of tumor suppressor genes in cell cycle control. Additionally, these two areas have linked the expression and activity of cyclin/cdk complexes to the regulation of transcription of factors required for cell cycle progression.

A. Retinoblastoma and the Cloning of the *RB1* Gene

As described in many previous reviews,[41,52,56,175] retinoblastoma (RB) is a pediatric cancer of the eye affecting 1 in 20,000 children up to the age of 3. The genetic predisposition of 40% of the patients led to Knudson's two-hit hypothesis.[82,83] Despite the clinically dominant manifestation of heritable RB, it became clear from cytogenetic studies that bilateral RB is due, in fact, to a recessive mutation and gave rise to the notion that the gene responsible for RB is a "tumor suppressor" gene.[18,37,38,84,190] Mutation of both alleles of the retinoblastoma susceptibility gene (*RB1*) is required for formation of RB tumors, although cytogenetic studies have also revealed the presence of additional mutations in almost all RB tumors.[42] A dramatic example is the presence of an amplified region of chromosome 6p present in greater than 70% of RB tumors as an iso6p variant.[165] Thus, retinoblastoma, which is generally a highly aggressive, metastatic tumor when no longer contained within the limiting confines of the vitreous of the eye (e.g., see Ref. 48) resembles other cancers in accumulating mutations at several genetic loci.

Gross deletions on chromosome 13 afforded a marker to attempt isolation of the *RB1* gene. Subsequent to identifying the close linkage to the esterase D gene,[163,164] T. Dryja generated a number of probes very close to *RB1*. One of these was in the *RB1* gene itself and facilitated the cloning of *RB1*.[39] As was demonstrated subsequently, most of the mutations in *RB1* are not observable at the cytogenetic level but, rather, involve point mutations, small deletions or insertions, or skipping of specific exons. The reader is referred to additional reviews for more specific details.[40,55,91,187]

Characterization of *RB1* revealed that the gene encodes a nuclear phosphoprotein (pRB) of approximately 110 kDa.[93] Surprisingly, pRB message and protein can be detected in almost all cell types despite the restricted number of tissues that are affected by *RB1* mutations.[5,39] For example, despite the very high levels of RB protein in lymphoid cells, patients with bilateral RB have

no greater probability of developing leukemia than the general population.[139] These data suggested that in some tissues, redundant mechanisms may overcome the loss of pRB. Furthermore, the timing of the appearance of RB tumors suggested that pRB plays an important role in the development of some tissues.

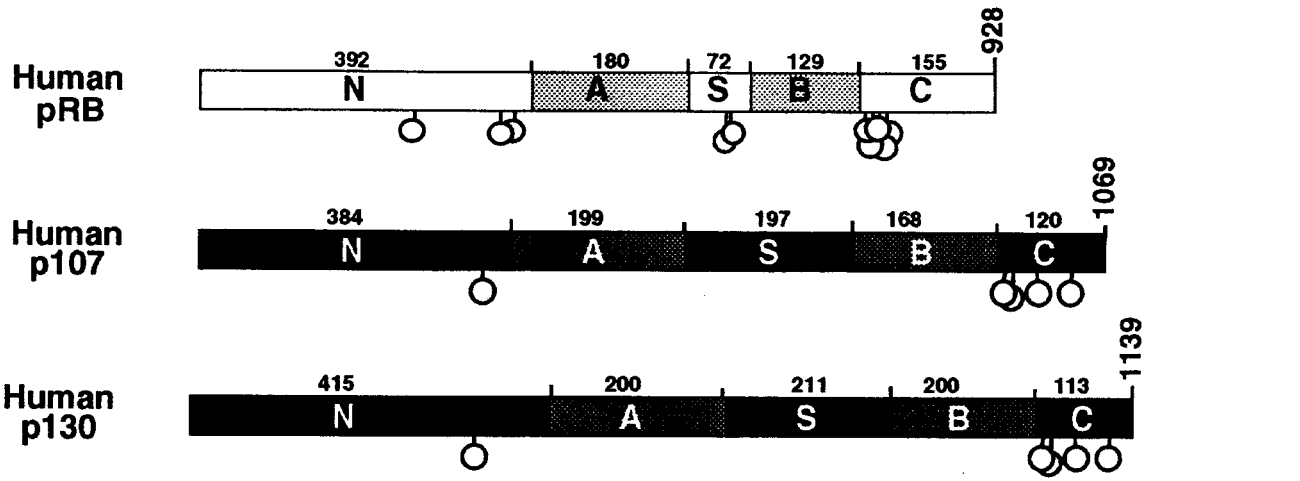
B. Cell Cycle-Dependent Phosphorylation of the Retinoblastoma Protein

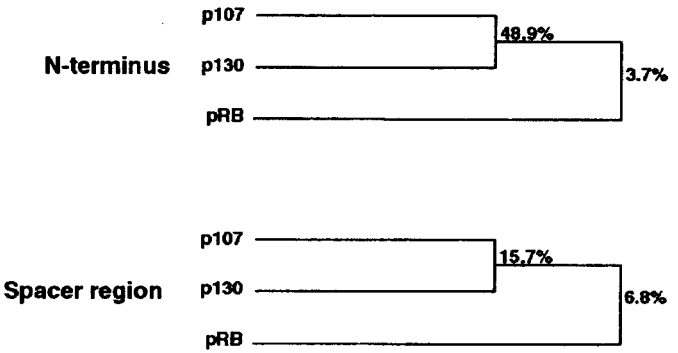
Whereas the levels of pRB do not substantially fluctuate during progression through the cell cycle, several groups demonstrated that pRB is posttranslationally modified in a cell cycle-dependent manner.[9,13,21,117] Specifically, in quiescent cells or in cells in G_1, pRB is present as a relatively under (hypo) phosphorylated species, migrating on SDS-PAGE gels as a single species with an apparent molecular weight of 110,000. As cells progress through G_1 toward S, pRB begins to migrate at successively high molecular weights (112,000 to 116,000) due to the protein becoming increasingly hyperphosphorylated. As cells pass through mitosis, pRB is quickly dephosphorylated and again appears as a hypophosphorylated protein in these early G_1 cells.[108]

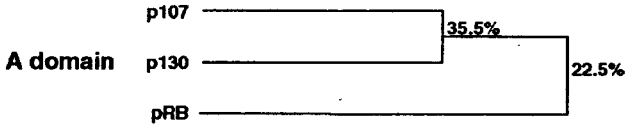
C. The Transforming Proteins of the DNA Tumor Viruses Complex pRB and pRB-Related Factors

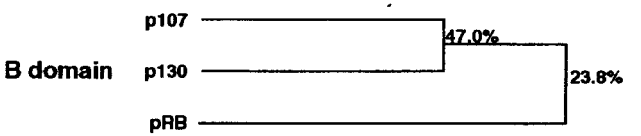
The observation that was essential for understanding at least one important function of pRB came from groups studying the DNA tumor viruses, simian virus 40 (SV40), adenovirus, and human papilloma virus (HPV). Although they exhibit very distinct features, all three of these viruses are able to induce quiescent cells to enter and progress through the cell cycle (for review see Ref. 122). Infection by SV40, adenovirus, or HPV leads to very early expression of their respective viral-transforming proteins, large T antigen (T_{Ag}), E1a, and E7. From co-immunoprecipitation assays, it was clear that these viral proteins interact with a number of nuclear factors in the host cells.[57,113,189] One of these factors, previously designated p105, is the retinoblastoma protein.[20,28,129,179] Despite having many distinct characteristics, T_{Ag}, E1a, and E7 all have a consensus sequence, termed the "LxCxE" motif, [28,130] in the region that is required for their transforming properties.[35,101,102,121,138,180] Mutation of this sequence results in loss of pRB binding and abrogates cellular transformation by T_{Ag}, E1a, and E7. Together these data implicated pRB in the negative regulation of cell cycle progression. Additionally, co-immunoprecipitation assays demonstrated that only the hypophosphorylated form of pRB bound to T_{Ag},[107] supporting the hypothesis that the hypophosphorylated form of pRB is the active species.

Mutation of the "LxCxE" motif in the transforming viral proteins abrogated their binding not only to pRB but also to at least two additional nuclear proteins that migrated close to pRB on SDS-PAGE, p107 and p130. Binding of these proteins to the conserved "LxCxE" sequences in T_{Ag}, E1a, and E7 suggested that p107 and p130 have structural features similar to pRB; the cloning of p107 [33] and p130[54,100,112] confirmed this hypothesis. All three pRB family proteins have two regions of relatively high similarity, termed A and B, which together form the "pocket domain" (see Figure 2; Refs. 67, 74). This pocket domain was originally defined by deletion analysis of pRB.[67] Small or gross deletions of sequences flanking the









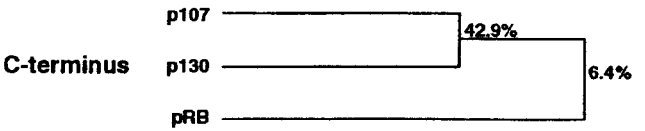


FIGURE 2. General structure of the pRB family proteins. The pRB family proteins, pRB, p107, and p130, contain two highly conserved domains, termed A and B, that together form the "pocket", defined as the region required for E1a binding. Regions flanking the pocket, the N and C terminal domains, as well as a spacer (S) region, are highly conserved between p107 and p130, but less so with pRB. The conserved spacer region of p107 and p130 mediates cyclin A and E binding, while the C terminal domain of pRB is involved in the *c-abl*/pRB interaction. Numbers above the various domains indicate the number of amino acids in those domains, while circles below represent Ser or Thr residues contained within consensus sequences recognized and phosphorylated by cyclin-dependent kinases. The similarity between individual domains of pRB, p107, and p130 at the amino acid level are indicated below the model. The overall similarity between pRB and p130 is 22% and between p107 and p130 is 50%.[100]

A and B domains have no effect on the binding of pRB to E1a, while any mutations in these regions abrogate binding. Interestingly, all naturally occurring mutations in pRB reported to date affect one or both of these domains.

Whereas the A and B domains are similar among all the pRB family proteins, the sequences flanking them are relatively distinct in pRB compared with p107 and p130. In contrast, p107 and p130 are strikingly similar to each other except for their N-termini. These differences in the primary structure of pRB relative to p107 and p130 are reflected by a number of important functional differences. One distinct characteristic is the ability of p107 and p130, but not pRB, to form physical complexes with cyclin E or cyclin A.[31,34,54,100] These interactions are mediated by sequences in the spacer regions between the A and B domains of p107 and p130.

The kinetics of the cell cycle-dependent phosphorylation of p107 and p130 are very similar to that of pRB. In quiescent mouse NIH3T3 fibroblasts or human glioblastoma T98G cells both proteins become phosphorylated approximately 8 h after serum stimulation.[3,111] Similarly, in a synchronized rat L_6 myoblast population, only the hyperphosphorylated form of p107 is present at mitosis. As these cells enter G_1, p107 is rapidly dephosphorylated and at the G_1/S boundary, hyperphosphorylated p107 is observed again.[78]

Initial reports indicated that the levels of pRB varied only about twofold during the course of the cell cycle.[117] However, as is observed for many cell cycle regulatory proteins (e.g., c-myc,[142] pRB, and p107), levels appear to be sharply increased in cells induced to enter the cell cycle following a mitogenic stimulus. For example, following stimulation of resting T cells with PHA, pRB, and p107 levels increase 4- to 6-fold after 18 and 24 h, respectively.[119] These strong inductions potentially occur only following a strong mitogenic stimulus, whereas in continuously cycling cells only limited fluctuations in the levels of these two proteins might be expected.

In contrast, dramatic changes in the level of expression of all three pRB family proteins have been observed as cells exit the cell cycle due to nutrient depletion or in order to terminally differentiate. For example, differentiation of myoblasts into myotubes is generally accompanied by increased levels of pRB,[29,78] and pRB levels are markedly increased during retinoic acid (RA)-induced differentiation of the embryonal carcinoma (EC) line P19.[159] Cell cycle exit following serum starvation or during terminal differentiation is generally accompanied by reciprocal changes in the levels of p107 and p130. In mouse embryo fibroblasts, for example, levels of p130 increase as cells reach a quiescent state after serum starvation.[16] More dramatically, p130 levels are strongly induced during L_6 and C_2C_{12} myoblast differentiation or RA-induced differentiation of P19 cells.[17,43,78,156] In L_6 myoblasts, an 8- to 10-fold induction of p130 is observed, while p107 levels are reduced 2- to 4-fold.[78] In all of these cell culture systems, pRB, p107, and p130 become predominantly hypophosphorylated in the quiescent and/or terminally differentiated cells.[13,78]

IV. INHIBITION OF CELL CYCLE PROGRESSION BY THE pRB FAMILY PROTEINS

Because either inactivation of the pRB family by the transforming proteins from DNA tumor viruses or loss of pRB during retinal development leads to uncontrolled cellular proliferation, it was anticipated that replacement of pRB in pRB-deficient cells might lead to cessation of proliferation. Wild-type pRB was first reintroduced into

the pRB-deficient human retinoblastoma tumor line, WERI-RB27, and human osteosarcoma cell line, SAOS-2.[68] Expression of the exogenous pRB in both these lines was initially reported to result in altered morphology, suppression of growth in culture, reduction of colony formation in soft agar, and absence of growth in nude mice. Whereas SAOS-2 cells have been shown in many labs to be particularly sensitive to reintroduction of functional pRB, other groups have had poor success in obtaining the same phenotype in pRB-reconstituted WERI-RB27.[128] Furthermore, expression of pRB in pRB-deficient cells (e.g., human RB lines, Y79[128] and J81, and the human cervical carcinoma C33A[196], among others) does not generally cause cellular quiescence. This failure to reverse the proliferative potential of pRB-deficient cells may reflect the fact that tumor cells, including those from RB tumors, have multiple genetic changes that contribute to their transformed phenotype. Replacement, therefore, of a single factor in these cells might be expected to have only a minor effect on the overall proliferative potential of these tumor lines. In addition, the presence of other cell cycle regulatory proteins, such as the G_1 cyclins and their associated kinases, would also be expected to regulate the activity of pRB in these pRB-reconstituted lines. Thus, while progression through the G_1 might be slightly delayed in these cells, overall cell cycle progression would be expected to persist. This hypothesis is supported by experiments employing a pRB protein that is mutated at eight potential phosphorylation sites and that is refractory to hyperphosphorylation.[50,51] When expressed in T cells, this mutant pRB (Δp34) was able to repress activated transcription by Elf-1 during S phase, while wild-type pRB became hyperphosphorylated and was inactive.[174] Δp34 has been used successfully to halt proliferation of normal smooth muscle cells.[12] Localized adenovirus-mediated introduction of Δp34 resulted in a strong reduction of smooth muscle proliferation *in vivo* following balloon angioplasty at arteriosclerotic sites in rats and pigs. Thus, the ability of pRB to suppress proliferation may critically depend on the individual cell, the level of exogenous pRB expression achieved, the level and activity of the cyclin/cdk complexes, and the role that pRB plays in the normal development and proliferation of the tissue.

It has also been suggested that many cells can tolerate the loss of pRB due to functional redundancy of the pRB family proteins, specifically p107 and p130. However, for many tumors such redundancy is not apparent. For example, although both the retinoblastoma tumor cell line, Y79, and human cervical carcinoma, C33A, express high levels of p130 and p107 (O. Wiggan and A. Sidle, unpublished observation), their presence is clearly not sufficient to reverse the transformed phenotype of these cells.

Redundancy of the pRB family proteins has been implied, however, following production of nullizygous pRB mice.[72,92,181] These pRB-deficient mice die near E14 of gestation due to central nervous system defects and apparent deficiencies in hematopoeisis. However, many other tissues develop in these mice despite the lack of functional pRB. Furthermore, chimeric mice in which the contribution of pRB-deficient cells approaches 80 to 90% generally reach term and have limited if any defects.[181] Because many tissues clearly tolerate the lack of pRB, p107 and p130 may compensate for its absence. Compensation by p107 for the loss of pRB, for example, was implied following studies using myoblast lines derived from pRB-deficient mice.[147] In these pRB-deficient myoblasts, p107 levels are apparently increased and overcome the loss of pRB during myoblast differentiation into immature muscle (myotubes). Complete compensation by p107 for pRB was not achieved in these lines, however, because

the terminally differentiated myotubes could respond to serum stimulation by reentering the cell cycle and reaching S phase. Taken together, the question of functional redundancy of the pRB family proteins remains controversial.

While there have been no clearly identified mutations of p107 and few mutations of p130 in human cancers,[15,33,54,100,172] p107 and p130 have growth-suppressive activity in certain cell types. Transfection of p107 induces growth suppression in at least one pRB-deficient cell line that is unaffected by reconstitution with pRB. Specifically, reintroduction of pRB into the pRB-deficient human cervical carcinoma, C33A, only weakly blocks G_1 progression, while p107 expression causes G_1 arrest.[196] The mechanism for p107 growth suppression may be distinct from that used by pRB, however, because p107 may prevent cell cycle progression by complexing and inhibiting the kinase activity of cyclin E/cdk2 and cyclin A/cdk2 complexes. This model is supported by the observation that p107 growth suppressive activity can be mediated by its spacer domain, a cyclin-binding region that pRB lacks.[194] p130 also causes arrest of at least one cell line that is refractory to pRB and p107, specifically the human glioblastoma line T98G.[15] These data support the notion that pRB, p107, and p130 have distinct properties and may have distinct roles in control of cell proliferation and/or cell cycle exit.

V. ACTIVITY OF THE pRB FAMILY PROTEINS IS REGULATED BY CYCLINS

A. pRB, p107, and p130 Contain Consensus Sequences Recognized by Cyclin-Dependent Kinases

The cell cycle-dependent phosphorylation of pRB, p107, and p130 suggests that they are targets of cyclin/cyclin-dependent kinase (cdk) complexes. This hypothesis was supported further by the observation that pRB, p107, and p130 contain numerous phosphorylation consensus sequences (Ser/Thr-Pro-X-Basic; for reviews see Refs. 99, 123, 140) recognized by cyclin-dependent kinases (cdk's). Phosphorylation by this class of kinases was confirmed directly using immunoprecipitated mammalian $p34^{cdc2}$.[96,103] Because pRB, p107, and p130 become hyperphosphorylated during G_1, it is likely that the G_1 cyclins, cyclins D1, D2, D3, and/or E, in association with their respective kinases, would be the important complexes mediating phosphorylation of the pRB family proteins. Co-(over)expression assays have shown that all of these G_1 cyclins, as well as cyclin A, can recognize and phosphorylate pRB, p107, and p130.[8,23,32,66,77]

Several investigators have suggested that *in vivo*, the D-type cyclins, in association with cdk4 or cdk6, may be the principal complexes phosphorylating and, thus, inactivating the pRB family proteins. First, cdk4 and cdk6 show a distinct preference for pRB as a substrate[2,109,116] relative to the traditional substrate used in these kinase assays, histone H1. Second, Rat-1 cells, in which cyclin D1 or cyclin E can be conditionally expressed, show distinct differences in their ability to induce pRB phosphorylation.[145] Specifically, following serum stimulation, conditional expression of cyclin D1 results in the immediate hyperphosphorylation of endogenous pRB and an acceleration of the G_1 to S transition. In contrast, despite a similar acceleration into S, conditional expression of cyclin E was not accompanied by an immediate hyperphosphorylation of pRB. Rather, pRB became hyperphosphorylated just prior to entry into S. Failure of cyclin E to phosphorylate pRB in these cells was not due to lack of kinase activity associated with cyclin E because immune complexes containing cyclin E isolated from cells early in G_1 but prior to hyperphos-

phorylation of pRB had demonstrable associated kinase activity. Finally, experiments examining the cell cycle-dependent phosphorylation of p107 determined that p107 first becomes hyperphosphorylated when cyclin D-associated kinase activity appears.[3] Nevertheless, it has been proposed that while cyclin D complexes mediate initial pRB hyperphosphorylation, this activity does not result in a fully phosphorylated protein.[59,118] Only later in the cell cycle, when cyclin E activity is present, is maximally phosphorylated pRB observed.

Taken together, these data support a model in which the initial hyperphosphorylation of pRB family proteins is mediated by the D-type cyclins complexed to cdk4 or cdk6. Later, as cells progress through the cell cycle, other cyclin/cdk complexes, specifically cyclin E/cdk2 and cyclin A/cdk2, may contribute to maintaining the pRB family proteins in a hyperphosphorylated state. Given that the apparent half-life of phosphate groups on pRB is on the order of 15 min[13] due, presumably, to constitutive phosphatase activity, maintaining pRB in its hyperphosphorylated form between late G_1 and M would require that cyclin/cdk kinase activity be present throughout this period. It should be mentioned here, however, that a number of studies have demonstrated that active, hypophosphorylated pRB and p107 may persist throughout the cell cycle (see below).

B. Inhibition of Cell Cycle Progression by the pRB Family is Reversed by Cyclin Expression

As described earlier, the human osteosarcoma line, SAOS-2, is arrested in G_1 following ectopic expression of pRB, p107, and p130, whereas proliferation of the human cervical carcinoma, C33A, is repressed only by p107. Co-expression of cyclin D1, E, or A with pRB in SAOS-2 reverses the antiproliferative effect of pRB.[66] The initial report of this assay demonstrated that only cyclin E and cyclin A caused hyperphosphorylation of pRB and that this inactivation of pRB was presumably sufficient to reverse its antiproliferative effect.[66] Because cyclin D1 did not appear to cause hyperphosphorylation of pRB, it was suggested that this cyclin reversed the effect of pRB in SAOS-2 cells by an alternative mechanism (see below).

In contrast, repression of SAOS-2 proliferation by p107 was refractory to addition of cyclin A and only weakly reversed by cyclin E.[3,196] Only cyclin D1 or D3 fully rescued SAOS-2 cells from repression by p107.[3] Furthermore, a mutant p107 molecule that was deleted at its C-terminus (domains B and C) but retained cyclin A and E binding activity could arrest both SAOS-2 and C33A cells in G_1. When a p107 mutant, deleted for its N-terminal domain and that failed to bind cyclins A or E, was expressed, G_1 arrest was observed in SAOS-2 cells but not C33A.[194] Finally, unlike pRB, only expression of cyclin D1, but not cyclin A or E, increased the amount of hyperphosphorylated p107 in C33A cells.[3] These results suggest that p107 uses two independent mechanisms to mediate growth suppression. One mechanism is analogous to that used by pRB and requires an intact pocket domain (A, B, and C domains). Presumably, this mechanism is mediated through repression of E2F activity. p107 can also repress proliferation via its spacer region, this region binding to and apparently repressing the kinase activity of cyclin A and cyclin E-containing complexes.

C. The pRB Family Proteins Form Stable Complexes with the G_1 Cyclins and Cyclin A

Two lines of evidence suggest that the D-type cyclins might complex with the pRB family proteins. First, the N-termini of

cyclins D1, D2, and D3 all contain the "LxCxE" consensus sequence[53,98,125] that is required for T_{Ag}, E1a, and E7 binding to pRB, p107, and p130. Second, reversal of pRB repression of SAOS-2 proliferation by cyclin D1 is apparently not accompanied by hyperphosphorylation of pRB,[66] suggesting that cyclin D1 may affect pRB function in a manner distinct from mediating its hyperphosphorylation. When expressed together at high levels in insect cells or when *in vitro*-translated proteins are allowed to bind to GST fusion proteins, cyclins D2 and D3, but not cyclin D1, associate with pRB or p107.[32] When cyclins D2 and D3 are co-expressed with cdk4, hyperphosphorylation of pRB is observed but cyclin D2- or D3-pRB co-complexes are undetectable. On the other hand, another group demonstrated by co-immunoprecipitation assays that cyclin D1 could form co-complexes with pRB *in vivo*.[23] In this study, neither cyclin D1 nor cyclin D3 could mediate the hyperphosphorylation of pRB in SAOS-2 co-expression assays despite the reversal of pRB-mediated cell cycle arrest, potentially due to limiting amounts of cdk4 in these cells. Finally, cyclin D3 associates with both p107 and p130 *in vivo*.[78] Differentiation of L_6 myoblasts into terminally differentiated myotubes results in strong induction of both cyclin D3 and p130. Co-immunoprecipitation assays demonstrated that a significant proportion of the p130 was bound to cyclin D3/cdk4 in these cells. p107-cyclin D3 complexes were also observed in terminally differentiated myotubes.

The role for pRB family D-type cyclin complexes remains speculative. The association of cyclin D1 with pRB may represent a mechanism for controlling the activity of the cyclin/cdk complex, in which binding to pRB prevents the activation of kinase activity by cdk-activating kinases. Reciprocally, binding of the D-type cyclins may represent an additional mechanism for modulating pRB family function, specifically preventing their association with any number of transcription factors, such as E2F.

Cyclins E and A, on the other hand, can bind to the spacer region of both p107 and p130 but not pRB.[31,34,54,100] It was demonstrated further that the association of cyclin E or cyclin A with p107 occurs in a cell cycle-dependent manner.[94] Early in G_1, p107 is not complexed to either cyclin E or A as neither is expressed at this point in the cell cycle. As cells progress through mid and late G_1, p107 complexes containing cyclin E are observed and are then replaced by complexes containing cyclin A as cells traverse S phase. The association of p107 with cyclin A/cdk2 appears to inhibit kinase activity of this complex.[195] Inhibition of kinase activity occurs by virtue of a sequence in the spacer region of p107, which is very similar to the cyclin-binding region of the important cyclin/cdk inhibitor p21. In fact, mutually exclusive binding of p107 or p21 to cyclin A/cdk2 co-complexes has been demonstrated.

The binding of cyclins E and A by p107 affords a potential mechanism used by this pRB family protein to inhibit cell cycle progression. Inhibition of kinase activity by p107 would be expected to prevent cell cycle progression because kinase activity of cyclin E/cdk2 and cyclin A/cdk2 complexes is required for the G_1 to S transition or progression through S phase, respectively. This prediction was confirmed by expressing a truncated version of p107, which lacks the B domain and the C-terminal portion. This mutant of p107, which cannot bind to factors such as T_{Ag}, E1a, and E2F, was capable of blocking cellular proliferation.[161,194]

VI. THE pRB FAMILY INTERACT WITH TRANSCRIPTION FACTORS ACTIVATED BY THE TRANSFORMING VIRAL PROTEINS

Subsequent to cloning of the pRB family proteins, a number of experimental strat-

egies led to the identification and/or isolation of nuclear proteins with which they interact. Given the consequences on quiescent cells of expression of the viral transforming proteins, E1a, T_{Ag}, and E7 following infection by adenovirus, SV40 and human papilloma virus, respectively, isolation and characterization of E2F has been the most intensely studied factor regulated by the pRB family proteins.

A. Cloning and Structural Features of the E2F/DP Family

The transcription factor E2F was originally identified as a cellular factor whose DNA-binding activity was stimulated after adenovirus infection of cultured cells. In mammalian cells, E2F is a heterodimeric, transcription factor composed of one of E2F-1, E2F-2, E2F-3, E2F-4, or E2F-5[4,10,44,61,65,71,75,97,146,151] and either DP-1 or DP-2.[47,184,193] E2F-1 was isolated by virtue of its ability to interact with pRB.[61,75,151] Subsequently, two closely related E2F's, E2F-2 and E2F-3, were cloned in low stringency screens of human NALM-6 cell, human fetal brain, and HeLa cell cDNA libraries.[71,97] E2F-4 and E2F-5 were isolated based on their ability to associate with p107,[4,44,65] p130,[146] or DP-1.[10]

The members of the E2F family contain several domains that are highly conserved (see Figure 3), including the basic helix-loop-helix (bHLH) and leucine zipper (LZ) domains. The LZ region, consisting of a 29-amino-acid-long hydrophobic heptad repeat, is located immediately carboxy terminal to the DNA-binding domain[44,95] and is essential for heterodimerization of E2F with the DP proteins.[62] A carboxy-terminal transactivation domain, which includes the binding motif for the pRB family proteins, has been mapped in human E2F-1.[61,75,151] The pRB family binding motif present in the transactivation domain does not appear to conform exactly to the "LxCxE" motif used by the transforming viral proteins and is also dependent on conserved residues that flank this motif.[149] Finally, an additional well-conserved sequence motif, named the "marked box", present between the LZ and the transactivation domain, is of unknown function.[97]

The first three members identified in the E2F family, E2F-1, E2F-2, and E2F-3, share the closest sequence similarity. All three possess a conserved N-terminal sequence adjacent to the DNA-binding domain that allows them to form complexes directly with cyclin A.[88,186] This region is absent from both E2F-4 and E2F-5.

E2F/DP heterodimers specifically bind to double-stranded DNA encoding the general consensus sequence, 5'-TTT(C/G)(C/G)CGC-3', which was originally identified in the promoter of the adenovirus E2 gene.[87,188] *In vitro*, the most stable complex between E2F-1/DP-1 and DNA is observed for the sequence TTTCGCGCCAAAA, which represents two overlapping, oppositely oriented sites.[173] The E2F-binding consensus sequences that are found in promoters of many growth regulatory genes have been demonstrated to be responsible for regulating activated transcription by E2F.[7,19,51,63,115,120,127,132,137,169,197]

Consistent with the notion that many of the basic proteins controlling the cell cycle are conserved, E2F and DP-1 homologues have been identified in a range of other species.[46,47,62,184,193] For example, *Xenopus laevis* homologues of DP-1 and DP-2 are encoded as maternally stored transcripts that are translated into protein during early development.[46] Interestingly, an E2F-like protein was recently identified in *Drosophila,* leading to speculation that a protein(s) similar to those of the pRB family may also exist in flies.[25,26]

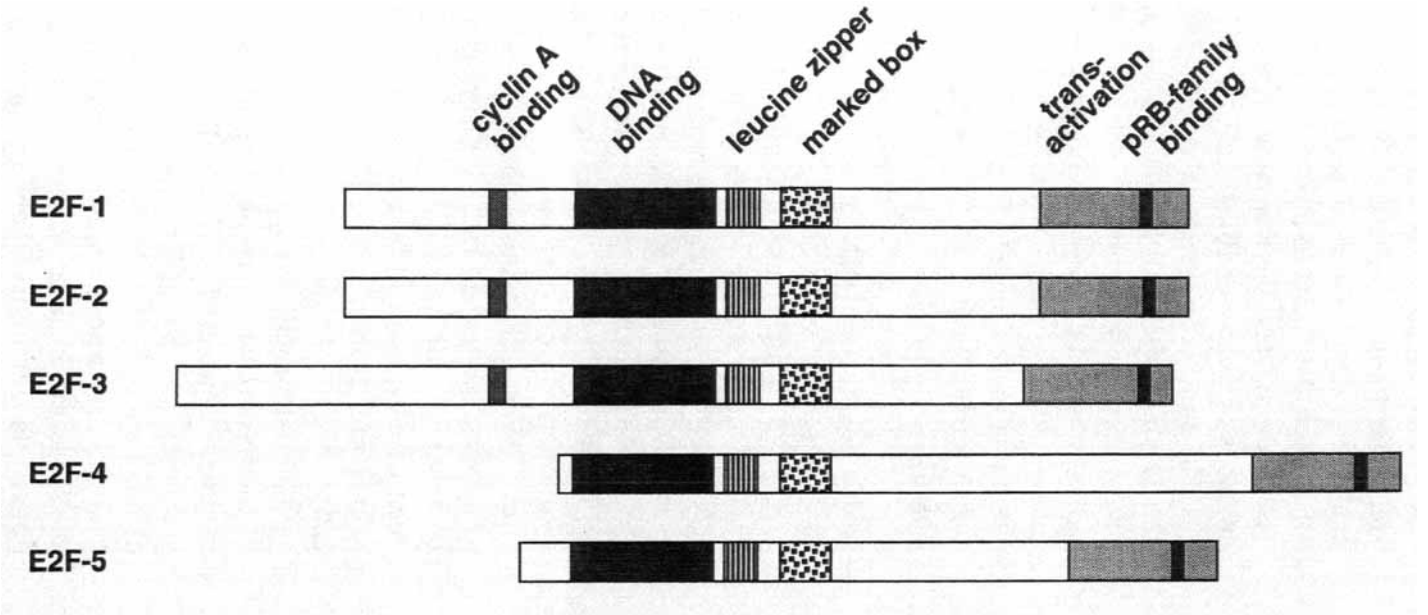


FIGURE 3. General structure of the E2F family of transcription factors. The five members of the E2F family of cell cycle transcription factors contain a number of highly conserved regions. These include the regions responsible for DNA binding, transcriptional activation, and a "leucine zipper" motif. The leucine zipper, located just C-terminal to the DNA binding domain, is essential for heterodimerization with DP family members. The transactivation domain is located at the C-terminus and contains within it a region responsible for binding to pRB family members. E2F-1, -2, and -3 also have an N-terminal cyclin A-binding region that is absent in E2F-4 and E2F-5. The first three E2F family members identified, E2F-1, E2F-2, and E2F-3, bind preferentially to pRB, while E2F-4 binds pRB, p107, and p130 and E2F-5 binds p130. (Adapted from Reference 146.)

B. Cell Cycle-Dependent Regulation of the E2F/DP-1 Activity

1. Regulated Expression of the E2F Family

Expression of the E2F/DP family members can be observed in a wide variety of different tissues and cell lines. The levels of mRNA expression are highly variable and are, to some extent, tissue-restricted.[4,10,44,46,61,65,71,97,172,184,193] The different E2F's also have unique patterns of mRNA expression in different cell types during the cell cycle or as cells enter the cell cycle from a quiescent state. E2F-1, for example, was not detected in extracts of resting (G_0) primary human T lymphocytes[14] and is almost undetectable in G_0 and early G_1 of the cell cycle in serum starved cell lines. Synthesis of E2F-1 mRNA is induced in mid G_1 and reaches its highest levels in S phase.[75,146,151,160] An additional study using T cells confirmed the result examining E2F-1 expression and demonstrated further that both E2F-3 and E2F-4 have similar kinetics of induction, with sharp increases in expression occurring in S phase. In contrast to T cells, E2F-4 and E2F-5 transcripts in fibroblasts are abundant in G_0, with E2F-4 expression considerably higher than E2F-5 in these quiescent cells.[44,146] In mid G_1, expression of E2F-4 mRNA is only increased about 2- to 3-fold, whereas the level of E2F-5 is elevated about 12-fold. The levels for both transcripts decline with similar kinetics when cells enter S phase.[146]

The induction of DP-1 mRNA in mid G_1 is almost identical to the induction of E2F-4, although DP-1 mRNA levels do not decrease during S phase.[44,146] Thus, DP-1 expression overlaps with the expression of all E2F family members throughout the cell cycle as would be expected if DP-1 or DP-2 were not limiting factors for E2F activity.[184]

2. Regulation of E2F Activity during the Cell Cycle

Most of the E2F transcription factors were isolated by their ability to bind to the pRB family proteins (see Figure 3; Refs. 61, 75, 151). *In vivo*, however, these pRB-E2F interactions are relatively restricted, that is, pRB binds preferentially to E2F-1, -2, and -3,[97] p107 to E2F-4, and p130 to E2F-4 and E2F-5.[4,441,46,172] Binding of the pRB family proteins to a sequence in the transcriptional activation domain of E2F blocks transactivation by E2F.[1,8,27,36,51,60,64,148,161,172,191,192,196]

The association of the pRB family proteins with the E2F's was predicted to be modulated during the cell cycle because the activity of the pRB family proteins are regulated in a cell cycle-dependent manner (Figure 4). In its active, hypophosphorylated form, pRB sequesters E2F-1/DP-1 heterodimers, inhibiting activated transcription by these complexes.[27] As cells progress through G_1, pRB becomes inactivated by phosphorylation, E2F/DP-1 is released from the repressive effects of pRB and activated transcription occurs from E2F-site containing promoters. E2F-1-mediated activated transcription does not appear to persist throughout the remainder of the cell cycle, however, because, as demonstrated both *in vitro* and *in vivo*, cyclin A/cdk2-directed kinase activity reduces the affinity of E2F-1/DP-1 heterodimers for DNA containing E2F binding sites.[88,186] This reduction of E2F/DP activity is necessary for progression through the cell cycle because preventing cyclin A from binding to and mediating phosphorylation of E2F/DP prevents cells from passing through S phase.[89] Therefore, as cells

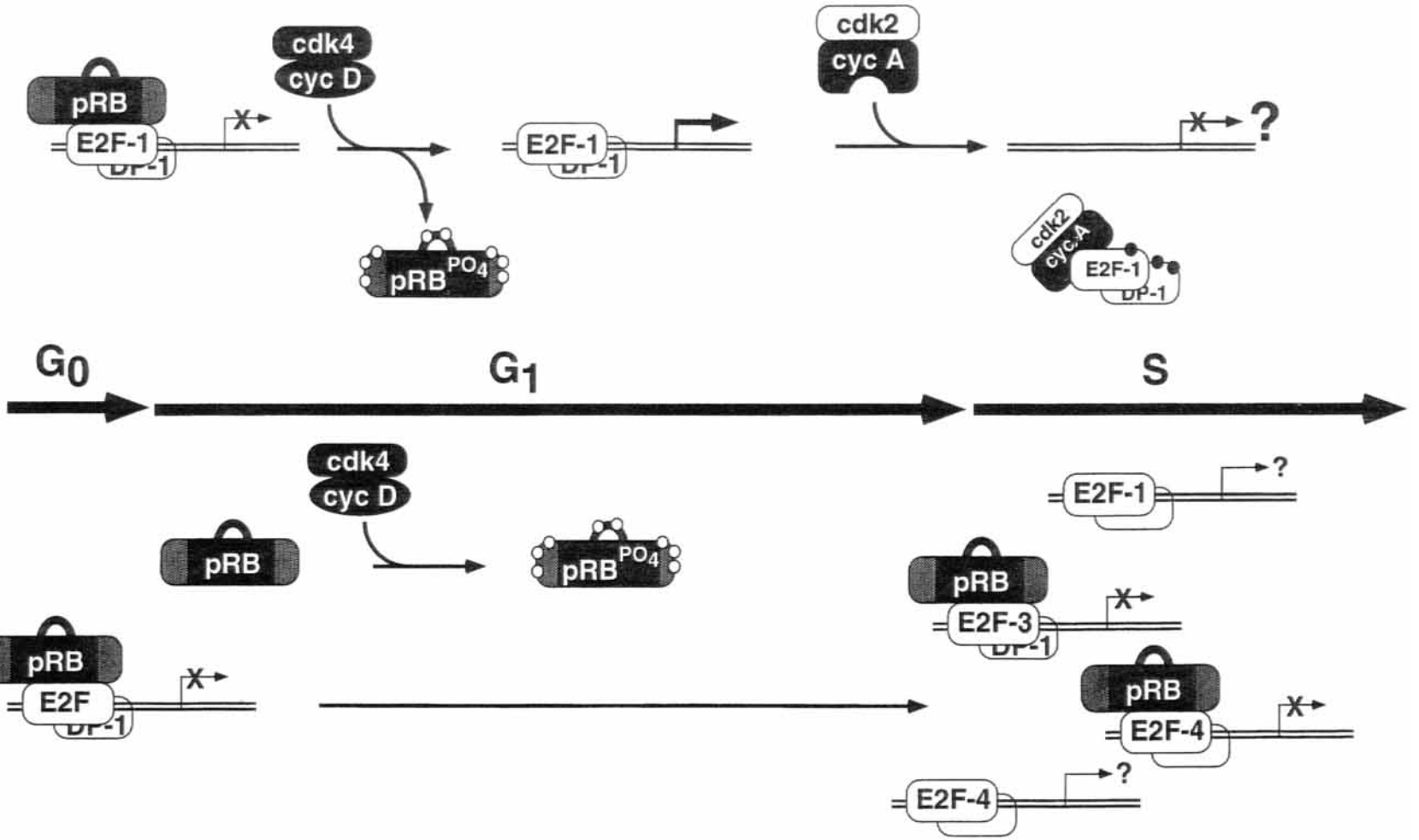


FIGURE 4. pRB and E2F-1 complexes during the cell cycle. One general model for the regulation of E2F-1 (and perhaps E2F-2 and E2F-3) is depicted in the scheme at the top of this figure. In quiescent cells or cells in early G_1, hypophosphorylated pRB binds to and represses activated transcription by the E2F family members, E2F-1, -2, and -3. As cells progress through G_1, pRB initially becomes hyperphosphorylated due to the kinase activity associated with the D-type cyclins, resulting in the release of E2F from pRB and stimulating activated transcription by E2F of factors required for progression through G_1 or for S phase. E2F does not continue to cause activated transcription during S phase, however, because expression of cyclin A in S phase results in hyperphosphorylation of E2F and/or its heterodimeric partner DP-1. This phosphorylation results in a weakening of the affinity of E2F-1/DP-1 for DNA and presumably prevents expression of E2F-dependent genes. This model predicts that E2F-1 is active only during a very discrete time in the cell cycle. Several observations (see text), however, indicate that pRB-E2F complexes persist, in fact, throughout the cell cycle. In addition, entry of some cell types into S phase from a quiescent state is accompanied by an induction of E2F-1, E2F-4, and pRB protein levels. In these cells, free E2F as well as co-complexes of E2F-3/pRB and E2F-4/pRB that can bind DNA containing E2F sites are also present during S phase. It was suggested that the pRB observed to undergo hyperphosphorylation may be newly synthesized protein. How these pRB/E2F co-complexes remain refractory to the cell cycle-dependent phosphorylation by cyclin/cdk complexes and the transcriptional activity of promoters regulated by these complexes remain undetermined.

progress through late G_1 where pRB is inactive, E2F-1/DP-1 would activate transcription of genes required for S phase. Once in S phase where cyclin A/cdk2 complexes accumulate, E2F-1/DP-1 becomes phosphorylated,[80] reducing its affinity for DNA and preventing activated transcription of E2F-regulated genes. This model predicts that E2F-1 would be transcriptionally active only transiently during the cell cycle, specifically during mid to late G_1 and in early S phase.

It may not, however, be possible to use this mechanism of regulation of E2F-1 by pRB as a general model for regulation of other E2F family members by pRB because pRB-E2F complexes, capable of binding DNA in electrophoretic mobility shift assays (EMSA's), can be observed throughout the cell cycle. In CV-1 cells synchronized in S phase by a double hydroxyurea (HU) block, E2F-pRB complexes capable of binding to E2F sites are present simultaneously with E2F-site binding complexes containing cyclin A.[148] This pRB-E2F complex persisted as cells were released from the HU block, with fluctuations in the levels of this complex due to fluctuations in the amounts of these proteins during the cell cycle. Furthermore, the authors suggest that the appearance of E2F complexes devoid of pRB results from new E2F synthesis rather than release of pRB-bound E2F. Another recent study also concluded that pRB-E2F complexes that could bind to DNA were present during S phase. Specifically, following PHA stimulation of resting human peripheral T lymphocytes, pRB-E2F complexes could not be detected in resting (G_0) or early G_1 cells. Not until S phase, when E2F-1, E2F-3, and E2F-4, as well as pRB and p107 levels are sharply increased, could "free" E2F complexes as well as E2F-3 and E2F-4 complexes containing pRB or p107 be detected.[119] The authors suggest that the presence of these new E2F complexes containing pRB and p107 during S phase is due to their *de novo* synthesis.

Several observations indicate that the cell cycle-dependent regulation of E2F-4 by p107 is more complex than that of E2F-1 by pRB. This greater complexity arises from the fact that p107 can form physical complexes simultaneously with cyclin A or cyclin E and E2F-4 and that the kinase activity of these cyclin/cdk co-complexes may be regulated by their association with p107. As depicted in Figure 5, p107 may bind to E2F-4 early in G_1, repressing E2F-4-mediated activated transcription. Like pRB, p107 is a target of cyclin D1/cdk4 kinase activity and hyperphosphorylation of p107 prevents its association with E2F-4. The E2F-4 released from p107 repression may activate transcription in a manner analogous to E2F-1 released from pRB repression.

Three observations make it clear, however, that the regulation of E2F-4 activity by p107 is more complex than this simple model predicts. First, as cells progress into late G_1 and then into S phase, p107 remains complexed with E2F and either cyclin E or cyclin A.[78,94,157] Second, in electrophoretic mobility shift assays, E2F-site binding complexes containing both p107 and cyclin A/cdk2 can be demonstrated,[11,78,126] despite being at a point in the cell cycle where p107 should be inactive (i.e., when cyclin A is expressed). Finally, after binding to p107 in its spacer region, cyclin A/cdk2 kinase activity becomes inhibited.[195] The cyclin A/cdk2 binding site in p21 is similar to the binding site in the spacer region of p107, and inhibition by p107 and p21 occur by their mutually exclusive binding to cyclin A/cdk2. This latter observation is at odds with the demonstration that after addition of ATP to reconstituted E2F-4/DP-1/p107/cyclin A/cdk2 complexes or to DNA-binding complexes isolated from the DLD-1 adenocarcinoma, p107, cyclin A and cdk2 are released from E2F-4.[167,195] This release

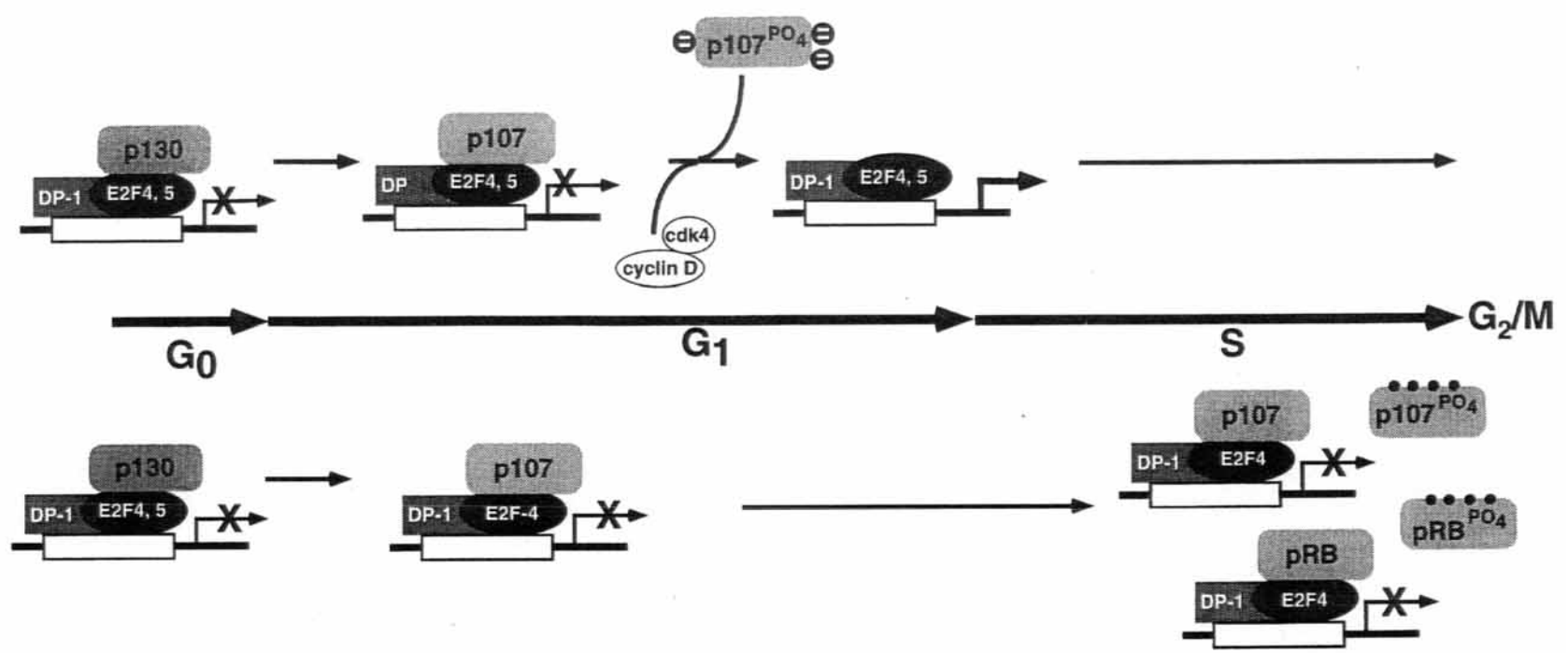


FIGURE 5. Cell cycle-dependent regulation of E2F activity by p107 and p130. Despite the similarity between pRB and p107 cell cycle phosphorylation and their ability to repress E2F-dependent activated transcription, the cell cycle profiles of their interactions with E2F family members suggest that they may play distinct roles in regulating cell cycle progression. In addition, although both p107 and p130 share a high degree of sequence similarity, similar cell cycle-dependent phosphorylation patterns and ability to bind to E2F-4 and E2F-5, they too may fulfill different functions. First, p130-containing E2F-complexes are predominant in G_0. As cells exit G_0 and progress into G_1, E2F complexes containing p107 are then found. Both the p130 complexes in G_0 and the p107 complexes in early G_1 are presumed to repress E2F-4- and E2F-5-mediated activated transcription. As cells progress through G_1, p107 is hyperphosphorylated following the expression of the D-type cyclins, resulting in its release from E2F-4 and/or E2F-5 and allowing activated transcription of a number of genes. As described above in Figure 4 for pRB, it has also been demonstrated recently that p107/E2F-4 co-complexes can be seen in cells in S phase. The same authors suggest that the hyperphosphorylation of p107 and pRB observed as cells progress through the cell cycle occurs only on newly synthesized protein. Furthermore, not depicted here but relevant to both schemes are studies that observed p107/E2F complexes capable of binding DNA also contain cyclin E (late in G_1) and cyclin A (during S phase). The transcriptional activity of promoters bound by these complexes remains undetermined.

of p107/cyclin A/cdk2 is accompanied by increased levels of "free" E2F-4/DP-1 complexes observed in electrophoretic mobility shift assays using an E2F-site probe, implying that the binding of p107 to cdk/cyclin complexes does not inhibit kinase activity.

Thus, there are several possibilities for the regulation of E2F-4 activated transcription during the course of the cell cycle. In the simplest model, E2F-4 is regulated by p107 in a manner analogous to E2F-1 regulation by pRB. However, this model does not take into account E2F-4 complexes that can bind DNA in the presence of cyclin A and p107. Potentially, a fraction of these E2F-4/p107 complexes persist throughout the cell cycle and the observed unphosphorylated p107 is due to newly synthesized protein.[3] Alternatively, a fraction of existing E2F-4/p107 complexes may be resistant to or protected from the effects of specific cyclin/cdk activity. Therefore, it will be important to take inventory of the different E2F-4/p107/cyclin/cdk complexes present during the cell cycle and determine their relative stoichiometry. For example, the presence and associated kinase activity of E2F-4/p107/cyclin E complexes binding to E2F sites remains relatively uncharacterized, and the levels of active and inactive E2F-site binding complexes are undetermined. Furthermore, the actual transcriptional activity of E2F-4 during the cell cycle remains to be determined.

Of all pRB family members, the least is known about how p130 interacts with E2F during the cell cycle. It is likely that p130 and p107 could be involved in very similar mechanisms of regulating E2F activity due to their interaction with a common subset of E2F proteins, E2F-4 and/or E2F-5, both of which lack the cyclin A binding site found in the E2F proteins complexed by pRB. Expression of p130, however, tends to be associated with quiescent cells and p130 expression and E2F-binding activity occur reciprocally to p107. For example, in serum-starved mouse embryo fibroblasts (MEF's), an induction of p130-containing complexes, at the expense of those containing p107, was observed.[16] A very recent study has also demonstrated that in resting human peripheral T lymphocytes stimulated to enter the cell cycle, the p130 present in E2F-4 complexes is replaced by p107.[119] However, p130-containing E2F-4 complexes have also been observed in both resting (G_0) and proliferating cells as well as in stimulated primary human T cells.[172]

3. E2F and pRB Families during Terminal Differentiation

Terminal differentiation is accompanied by exit from the cell cycle and a shift of the pRB family proteins to their active, unphosphorylated state. In addition, a number of studies have demonstrated that during the transition into a postmitotic state, "free" (i.e., E2F complexes not associated with pRB family proteins) E2F complexes are lost. The differentiated state is instead marked by the predominance of E2F-site binding complexes containing pRB and p130, while p107-containing complexes are reduced. For example, during differentiation of rat L_6 myoblasts into myotubes, an 8- to 10-fold induction of p130 and a 2-fold reduction of p107 was seen.[78] These changes in the relative levels of p130 and p107 were reflected by a quantitative shift of E2F-site binding complexes containing p107 to complexes containing p130 in both the rat L_6 and mouse C_2C_{12} cell lines.[78,156] A similar induction of p130 and appearance of p130-containing E2F-site binding complexes was observed during retinoic acid-induced differentiation of the embryonal carcinoma cell line P19 into postmitotic neurons.[17] So, as depicted in Figures 4 and 5, the E2F-com-

plexes in cycling cells appear to be dominated by p107 and pRB but in terminally differentiated and/or quiescent cells by p130 and pRB.

4. E2F Interacts with and Modifies the Activity of Flanking Transcription Factors

Many of the studies cited above examined the effects of E2F and pRB family proteins on simple promoter constructs containing single or tandem E2F binding sites. However, E2F sites are generally found in the context of considerably more complex promoters. For example, even in the adenovirus promoter, EIIa, the two E2F sites are flanked by an ATF site immediately upstream of the distal E2F site (e.g., see Ref. 105). This arrangement suggests that complexes containing E2F may act to modify the activity of the transcription factors that bind to sequences flanking these E2F binding sites. This notion has been demonstrated in a number of systems. When present in the context of an ATF site, the E2F site can behave as both a positive or negative regulatory element depending on the absence or presence of pRB, respectively.[177] When tandem E2F sites were placed upstream of the ATF-binding site, CAT activity of this reporter construct was increased relative to ATF alone. The addition of pRB caused repression of the E2F-ATF promoter construct to levels below that of those seen for ATF alone. The presence of E2F-pRB complexes in these "complex" promoters may also inhibit flanking transcription factors from interacting with the basal transcriptional machinery,[8,176] while simultaneously interacting itself with components such as TAF II_{250}.[152]

These data also predict that E2F and/or pRB may influence flanking transcription factors by direct physical interaction. This prediction was demonstrated recently for E2F and the transcription factor, Sp1, which were shown to bind to each other *in vitro* and could be co-immunoprecipitated from cells.[76,104] Analogous to ATF, E2F-1 enhances transcriptional activation by Sp1. Interestingly, only E2F-1, E2F-2, and E2F-3, but not E2F-4 or E2F-5, physically associate with Sp1 and, in the case of E2F-1, this interaction occurs in a cell cycle-dependent manner. A potential consequence of the interaction of Sp1 with specific E2F family proteins is that synergistic binding of these factors may occur on specific promoters, enhancing the probability that pRB regulates the expression of these genes by virtue of its specific interaction with E2F-1, E2F-2, and E2F-3.

The recruitment of pRB by E2F family proteins to specific promoters may also influence the binding of other pRB-associated factors to these sites. One important protein that may behave in this manner is c-abl.[178] C-abl is a ubiquitously expressed non-receptor tyrosine kinase (for review see Ref. 6), which possesses DNA binding activity that is regulated in a cell cycle-dependent manner.[79] Bacterially expressed pRB deletion mutants revealed that a 161 amino acid C-terminal domain of pRB was essential and sufficient for binding to c-abl *in vitro*. This *in vitro* pRB/c-abl interaction has been confirmed *in vivo* by co-immunoprecipitation assays. C-abl does not form complexes with p107 (J. Wang, personal communication) and probably does not bind p130 given its similarity to p107. The pRB/c-abl interaction was not affected by viral oncoprotein association with the pocket domain of pRB, and the pRB/c-abl interaction is totally independent of any known pRB pocket domain interactions. Analogous to its interaction with the E2F proteins, pRB-binding to c-abl is dependent on the phosphorylation state of pRB. C-abl associates

exclusively with the unphosphorylated form of pRB; pRB/c-abl complexes were detected in serum-starved, quiescent NIH3T3 cells, but not during S phase where pRB exists in its inactive, hyperphosphorylated state. The release of c-abl from pRB correlates with an increase in the kinase activity of c-abl during S-phase, consistent with a regulatory role for pRB in c-abl function. Additionally, c-abl autophosphorylation and its ability to phosphorylate an exogenous substrate was reduced 8- to 10-fold in the presence of a C-terminal pRB fragment.

Because various transcription factors can bind to different regions of pRB, complexes composed of a number of distinct transcription factors are predicted to exist on promoters containing E2F sites (Figure 6). The overall transcriptional activity of these higher-order complexes will depend on the different types of factors present as well as the phosphorylation state of those factors at any given time during the cell cycle. This model also suggests a mechanism whereby factors exhibiting nonspecific DNA binding, such as c-abl, may be targeted to specific promoters.

5. Oncogenic Activity of the E2F Family Proteins

A growing number of reports studying the deregulated expression of E2F family members suggest that they may function as oncogenes. Inducible expression of E2F-1 in low serum, using the tetracycline-controlled expression system or a modified metallothionine promoter system, lead to S phase entry followed by apoptosis.[141,150] Apoptosis was independent of serum concentration but was dependent on functional p53. Transfection or microinjection of an E2F-1 expression construct into quiescent rat embryo fibroblasts increased the number of cells entering S phase, as determined by BrdU staining.[73] This effect was accompanied by an increase in DHFR or TK promoter activity in the absence of serum and was dependent on the presence of the transactivation domain at the C-terminus of E2F-1. Similarly, infection of REF52 with adenovirus expressing E2F-1 increased transcription of endogenous genes required for the G_1/S transition and DNA synthesis such as PCNA, TS, DNA polymerase α, and E2F-1 and E2F-2.[22] More dramatically, deregulated expression of wild-type E2F-1 in rat embryo fibroblasts resulted in a transformed phenotype characterized by an increased number of spindle-shaped cells, a tendency to form foci in subconfluent cultures, lack of contact inhibition, faster doubling times, sustained growth in 0.1% serum, formation of colonies in soft agar, and tumor formation in nude mice.[158] These transformed cells had increased expression of E2F-regulated genes. This effect is downstream of the tumor-suppressive effects of pRB because deregulated expression in NIH3T3 fibroblasts of the E2F family members that specifically associate with pRB, E2F-1, E2F-2, and E2F-3 led to colony formation in a soft agar assay. Expression of a DNA binding-defective mutant of E2F-1 did not allow colony formation, while a pRB-binding mutant that retains transactivation function retained colony formation.

Different phenotypes and different effects on cell proliferation are observed with other deletion mutants of E2F. For example, transfection of mouse NIH3T3 cells with an amino terminal deletion mutant of E2F-1, which can no longer bind cyclin A, results in a partially transformed phenotype, with altered morphology and growth pattern, and loss of normal contact inhibition, but failure of growth in soft agar.[106] These cells were able to enter S phase in the presence of low serum, similar to those transfected with wild-

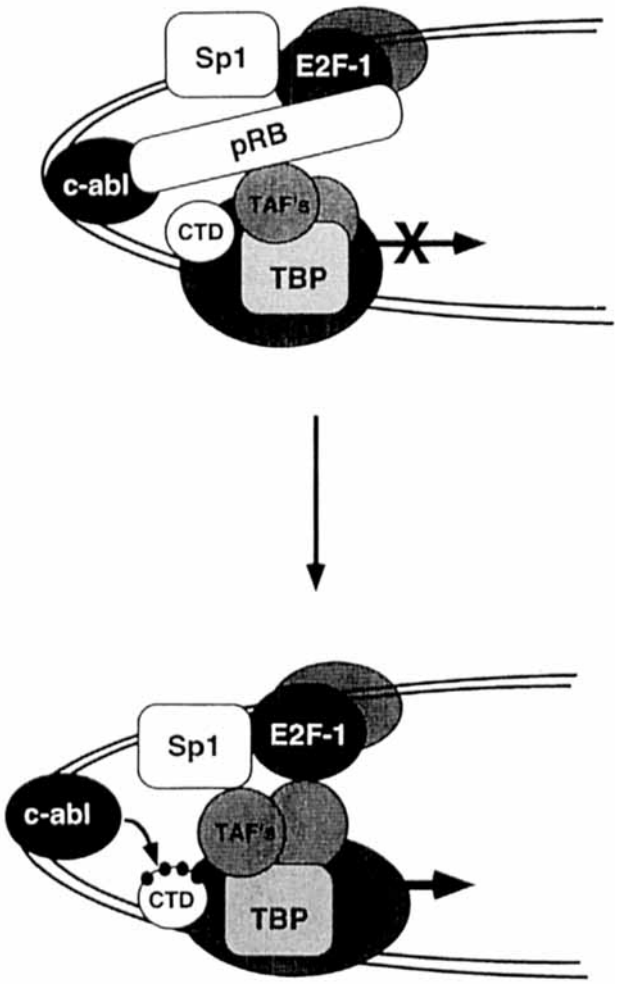


FIGURE 6. Regulation of promoter activity by the pRB and E2F family proteins. Interaction of pRB and E2F with various proteins that can localize to a single promoter suggest that a multisubunit complex may regulate these promoters. In one of many possible arrangements, both E2F and Sp1 could occupy specific sites in a promoter, the interaction between these two transcription factors potentially influencing their affinity for their respective sites. In quiescent cells or cells in early G_1, active, unphosphorylated pRB will also be present due to its association with E2F-1. Its presence may not only repress E2F-mediated activated transcription but could simultaneously repress activated transcription by Sp1, potentially by blocking access to the basal transcription machinery. The localization of pRB to promoters where E2F-1 binds could also facilitate the recruitment of c-abl by virtue of its binding to the C-terminal portion of pRB. Following a mitogenic stimulus, pRB would be inactivated due to the action of cyclin/cdk complexes. Once freed from the repressive effects of pRB, E2F and Sp1 could interact with the basal transcription machinery, synergistically driving transcription of specific genes. Additionally, c-abl, which was localized to this promoter due to its association with pRB, would also be poised to influence the rate of transcription by mediating phosphorylation of proteins assembled at the initiation site, such as the carboxy terminal domain (CTD) of RNA polymerase II (pol II).

type E2F-1, but both cells expressing either the cyclin A-binding mutant or wild-type E2F-1 proliferated poorly in low serum. However, only cells expressing wild-type E2F-1 retained contact inhibition and anchorage dependence for growth. This growth pattern suggests that increased expression of E2F-1 only partially replaces a serum-proliferative signal. It was noted, however, that cells expressing the amino terminal mutant demonstrated a twofold prolongation of S phase and increased sensitivity to S phase specific apoptosis-inducing agents relative to cells expressing wild-type E2F-1. These data indicate that cyclin A binding and the subsequent phosphorylation of E2F-1 and/or DP-1 is an important aspect of E2F-1 regulation and may provide a means by which E2F-1-regulated genes can be turned off as cells progress through S phase (see Figure 4).

E2F-4 appears to have only limited transforming activity. For example, expression of wild-type E2F-4 and DP-1 in Ha-Ras expressing REF cells led to full transformation, associated with increased E2F binding activity, with most E2F in the "free" form.[4] E2F-5 has not yet been reported to induce or cooperate with other genes to produce a transformed phenotype.

Overall, these studies show that E2F family members can function as oncogenes. Their transforming ability depends on the specific E2F member expressed, the specific cell type used for transformation, the presence of other regulatory proteins, and/or the level of expression achieved.

CONCLUSIONS

To conclude, it is clear that although a great deal is known about the molecular mechanisms that control the activity of the pRB family proteins, their involvement in tissue-specific cellular transformation remains undetermined. Likewise, although many different pRB-, p107-, and p130-containing E2F complexes have been defined both in cycling cells and in terminally differentiated cells, the transcriptional activity of these complexes at different stages of the cell cycle require considerably more investigation, particularly those involving p107 and p130. Although not discussed here, it is also clear that the pRB family proteins bind to and regulate the activity of other classes of nuclear factors. Ultimately, the tissue-specific consequences of losing functional pRB family members may be determined, at least in part, by these other interactions.

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