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Review

Regulation of E2F: a family of transcription factors involved in proliferation control

Adrian R. Black, Jane Azizkhan-Clifford *, [1]

Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263, USA

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Abstract

Members of the E2F family of transcription factors are key participants in orchestration of the cell cycle, cell growth arrest and apoptosis. Therefore, an understanding of the regulation of E2F activity is essential for an understanding of the control of cellular proliferation. E2F activity is regulated by the retinoblastoma family of tumor suppressors and by multiple other mechanisms. This review will describe our current knowledge of these mechanisms which together constitute a highly complex network by which the cell cycle and cellular proliferation can be controlled. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Precise control of cellular proliferation is essential for normal development and for prevention of proliferative diseases such as cancer. As such, the mechanisms underlying this control have been intensively studied and many of the factors involved are now known (King and Cidlowski, 1998). Over the past decade, the E2F family of transcription factors has emerged as a central component of this regulatory machinery and multiple mechanisms for its regulation have been identified. The best characterized of these involves the retinoblastoma family of tumor suppressors, and a coherent model has been developed to describe how these factors regulate E2F activity during the cell cycle (Bernards, 1997; Ewen, 1998). Although this model has proven highly valuable in the study of proliferation control, recent data indicate that regulation of E2F is more complex than originally envisioned. Therefore, this review will briefly summarize the currently established model of E2F regulation by the retinoblastoma family and will concentrate on other mechanisms by which E2F activity is modulated, thereby highlighting an increasingly intricate network by which cellular proliferation can be tightly controlled.

2. E2F-regulated genes

E2F[2] (E2 factor) is so named because of its initial identification as a cellular factor activated by E1a and involved in activation of the adenovirus E2 promoter (Kovesdi et al., 1986). The E2F binding site in the E2 promoter was identified as 'TTTCGCGC' and similar sequences were soon recognized in the promoters of the cellular *dihydrofolate reductase* (*DHFR*) and *c-myc* genes and shown to be involved in the regulation of their expression (Blake and Azizkhan, 1989; Hiebert et al., 1989). E2F sites were subsequently identified in a large number of cellular promoters. Most, if not all, of these are growth/cell cycle regulated genes that are involved in control of the cell cycle (e.g. *cdk2* and *4*, *cyclins A*, *D* and *E*) or DNA synthesis (e.g. *PCNA*, *DNA polymer-*

Abbreviations: DHFR, dihydrofolate reductase; RB, retinoblastoma; pRB, retinoblastoma protein.

* Corresponding author. Tel.: +1-716-845-3563; fax: +1-716-845-8857.

E-mail address: azizkhan@sc3101.med.buffalo.edu (J. Azizkhan-Clifford)

[1] Jane Azizkhan-Clifford has previously published under the name of Jane C. Azizkhan.

[2] Throughout this review, E2F family members will be referred to as E2Fs or E2F-1 to -6 and the term E2F, when unmodified, will be used generically to describe DNA binding E2F heterodimers.

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ase α, *ribonucleotide reductase*). Interestingly, E2Fs can regulate their own activity directly, through the presence of E2F sites in the promoters of *E2F-1* and *-2*, as well as indirectly, through the presence of E2F sites in the promoters of the E2F regulatory proteins, pRB, p107 and p130 (see Yamasaki, 1998 for a review).

E2F sites have been shown to be directly involved in the growth regulation of many genes. Generally, transcriptional induction of these genes following serum stimulation of serum-starved cells is seen in late G_1 and peaks in S-phase. However, these kinetics do not hold for all E2F-responsive genes, since some can be induced with immediate early kinetics (*c-myc*) or are induced in S-phase (*cdc2*, *cyclin A*).

3. The E2F family of transcription factors

E2F activity is comprised of a family of heterodimeric proteins of the basic helix–loop–helix class of transcription factors which recognize the consensus DNA sequence 'TTT(C/G)(C/G)CGC'. Each heterodimer contains one member of the E2F family and one member of the DP family of proteins. Like other basic helix–loop–helix transcription factors, E2Fs bind DNA as dimers and, while E2F and DP homodimers can bind DNA, this binding is very weak compared with that of E2F-DP heterodimers (Huber et al., 1993). E2F is evolutionarily conserved: it is found in insects as well as vertebrates (see Yamasaki, 1998 for references), and an E2F-like factor and E2F-binding sites have even been identified in yeast, although these remain to be fully characterized (Mai and Lipp, 1993; Zhang et al., 1999a).

In mammals, the E2F family has six identified members (E2F-1 to -6) which can be placed into three groups based on differing homology (Fig. 1). All family members have N-terminal DNA binding and dimerization domains, followed by a conserved region termed the 'marked box' which may be involved in dimerization and DNA bending (Vidal et al., 1996; Cress and Nevins, 1996). In all members except E2F-6, there is a C-terminal transactivation domain which is responsible for binding to the retinoblastoma family of tumor suppressors (see below). E2F-1 to -3 differ from E2F-4 to -6 in that they have a domain N-terminal to the DNA binding domain which binds to cyclin A, and E2F-6 differs from the other family members in that it lacks the transactivation domain. Two members of the DP family, DP1 and DP2 (also referred to as DP3 in mouse), have been identified and these have weak homology with E2Fs, especially in the DNA binding/dimerization domain. All E2F members can heterodimerize with both DP1 and DP2, allowing for the formation of at least 12 DNA-binding complexes. This number is likely to be increased by mechanisms such as alternate splicing which has been

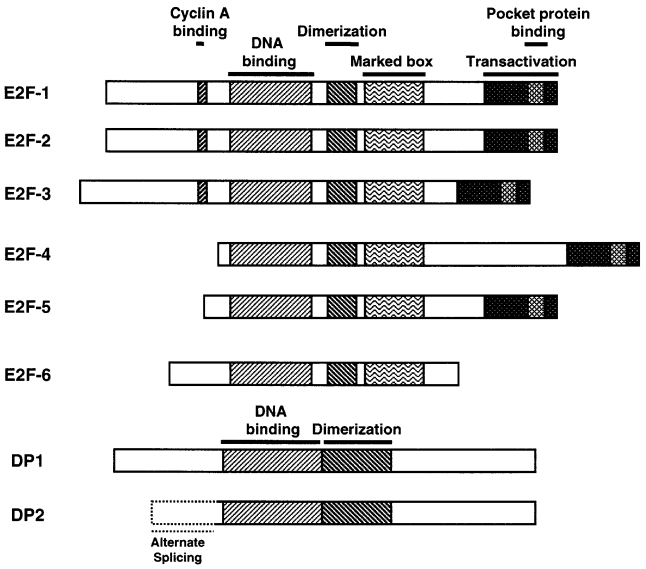

Fig. 1. The E2F family of transcription factors. Adapted from Sardet et al. (1997) and Cartwright et al. (1998).

demonstrated for DP2 (Ormondroyd et al., 1995; Zhang and Chellappan, 1995; Rogers et al., 1996). Differential properties of these E2F species would allow for regulation of E2F activity at multiple levels and, as will be discussed below, such mechanisms are emerging. The importance of multiple family members to regulation of E2F-dependent transcription is highlighted by the fact that the individual family members are conserved in mammals (cf. Yamasaki, 1998). Furthermore, in *Drosophila*, two E2F family members have been identified with drastically different transcriptional properties, one being an activating factor and the other being repressive (Dynlacht et al., 1994a; Ohtani et al., 1994; Sawado et al., 1998).

E2F can activate transcription by multiple mechanisms. The protein is able to directly interact with the basal transcription factors, TBP and TFIIH (Hagemeier et al., 1993; Emili and Ingles, 1995; Blau et al., 1996; Pearson and Greenblatt, 1997). In keeping with the roles of these proteins in pre-initiation complex formation and transcriptional elongation, E2F has been shown to enhance both the initiation and elongation phases of transcription (Blau et al., 1996). Additional basal components are likely to be involved in the transactivation by E2F, since it can mediate the cooperative assembly of a TFIID · TFIIA complex at the promoter in a manner that requires TBP-associated factors (Ross et al., 1999). E2F is able to bend DNA and this represents another mechanism by which E2F can activate transcription (Huber et al., 1994; Cress and Nevins, 1996). E2F can also effect transcription through its ability to bind to other transcriptionally active factors (see below), which provides additional levels of regulation by E2F.

4. The pRB pathway

The initial indication that E2F was involved in cell proliferation control came with its identification as a target for the product of the retinoblastoma tumor suppressor gene (Bagchi et al., 1991; Bandara and La Thangue, 1991; Chellappan et al., 1991; Chittenden et al., 1991). Intense study of this interaction has identified a pathway in which E2F is regulated through the cell cycle by its interaction with the retinoblastoma protein (pRB) and related proteins (see Sardet et al., 1997; Grana et al., 1998; Stiegler et al., 1998b; Lania et al., 1999 for reviews and references).

pRB is a member of a multigene family, containing two other identified members, p107 and p130. These proteins differ in their ability to interact with different E2F family members. Whereas pRB binds to E2F-1, -2, -3 and -4, p130 and p107 interact with only E2F-4 and -5. pRB, p107 and p130 bind E2F through a structure, termed the pocket, which is comprised of non-contiguous regions near their C-terminus. Since this pocket is central to many of the interactions of these proteins, they are collectively known as pocket proteins.

Cell cycle regulation of E2F-dependent transcription is mediated through the cell cycle by phosphorylation of pocket proteins. In G_0 and early G_1, pocket proteins are hypophosphorylated, and only these forms can interact with E2Fs. Binding to pocket proteins masks the transactivation domain of E2F and, thus, blocks the ability of E2F to activate transcription. As will be discussed below, pocket proteins also act as transcriptional repressors which further contribute to the silencing of E2F-site containing genes in G_0 and early G_1. In mid G_l, pRB is phosphorylated first by cyclin D · cdk4/6 and then by cyclin E · cdk2. Since these hyperphosphorylated forms of pRB can no longer bind E2F, E2F is released from the inhibitory complexes, resulting in an accumulation of 'free' E2F and activation of E2F-site containing genes in mid–late G_1. Interaction of p107 and p130 with E2F is regulated similarly.

Phosphorylation of pocket proteins is regulated by an array of cyclin-dependent kinase inhibitors, which are themselves regulated by a vast number of growth-regulated signals (see Peter, 1997 for a review). A classical example of such regulation is seen in the G_1 checkpoint induced by DNA damage (Chiarugi et al., 1994; Sladek, 1997). This is mediated, in large part, by the p53-dependent induction of $p21^{WAF1/CIP1}$. The resultant high levels of $p21^{WAF1/CIP1}$ prevent cyclin · cdk phosphorylation of pocket proteins, which become hypophosphorylated, interact with E2F, and inhibit transcription of E2F-regulated genes. However, it should be noted that cyclin-dependent kinase inhibitors and the pRB pathway interact at multiple levels; for example, $p21^{WAF1/CIP1}$, $p27^{KIP1}$ and $p57^{kip2}$ are also involved in formation of cyclin · cdk complexes (LaBaer et al., 1997), and the promoters for $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ are E2F-regulated (Deleu et al., 1998; Gartel et al., 1998; Hiyama et al., 1998).

Although pocket proteins are phosphorylated by cyclin-dependent kinases in G_1, complexes of E2F with all three pocket proteins persist into S-phase. In the case of p130 and p107 these complexes can contain cyclin A · cdk2 or cyclin E · cdk2. The function of these complexes is unclear and they may not be directly associated with transcription, since they have been found in the cytoplasm (Verona et al., 1997) and have been proposed to be involved in inhibition of cyclin-associated kinase activity (Woo et al., 1997).

The central role of the pRB pathway in regulation of cell proliferation is highlighted by the fact that it is a target of the immediate early gene products of several DNA tumor viruses, specifically simian virus 40 large T antigen, papilloma virus E7, and adenovirus E1a. These oncoproteins interact with the pocket and disrupt the interaction of E2Fs and pocket proteins, thereby activating E2F-dependent transcription (Nevins, 1994; Ewen, 1998). Furthermore, mutations in these immediate early proteins that abrogate their interaction with pocket proteins also abolish their transforming ability, suggesting a role for E2F in transformation.

In addition to the multiple levels of regulation of E2F described in this review, the interaction of pocket proteins with many other cellular proteins adds further complexity to this system of cell growth control (e.g. Larminie et al., 1997; Tevosian et al., 1997; van Wijnen et al., 1997; Voit et al., 1997; Sellers et al., 1998; Yee et al., 1998).

5. E2F as a positive and negative regulator of cell proliferation

The disruption of E2F control mechanisms in most, if not all, human cancers (Sherr, 1996), and its targeting by viral oncoproteins, argues that E2F plays a critical role in regulation of cell growth. This has been directly demonstrated by the ability of E2F-1, -2 or -3 overexpression to transform cells (Singh et al., 1994; Xu et al., 1995). Furthermore, DP1 and DP2 can transform cells in conjunction with activated ras (Jooss et al., 1995). E2F overexpression can also drive quiescent cells into S-phase. Interestingly, different E2Fs have different effects on the cell cycle/growth state of cells. E2F-1, -2 and -3, but not E2F-4 or -5, have been shown to induce S-phase in serum-starved or p16-arrested Rat1 fibroblasts or serum-starved REF52 cells (Lukas et al., 1996; DeGregori et al., 1997). Such effects may be cell type-specific since (a) overexpression of E2F-4 or -5 in conjunction with DP1 was shown to drive quiescent Rat1 but not REF52 cells into S-phase (Lukas et al., 1996; DeGregori et al., 1997), and (b) E2F-1, but not

E2F-3, was able to induce S-phase in growth factor-deprived pregranulocytes (Strom et al., 1998).

Although the above studies demonstrate that E2Fs can act as oncogenes, these factors have also been shown to have characteristics of tumor suppressors. *E2F-1* knockout mice have an increased incidence of tumors (Field et al., 1996; Yamasaki et al., 1996) and E2F-4 mutations are found in a high percentage of primary tumors (Souza et al., 1997). Furthermore, abrogation of E2F function using dominant negative E2F-2 or DP1 mutants can lead to transformation (Jooss et al., 1995; Bargou et al., 1996). These apparently opposing roles of E2Fs are likely to reside in their transcriptionally repressive and apoptotic functions which are discussed below.

6. E2F and apoptosis

In addition to modulation of cell growth and the cell cycle, effects on apoptosis also appear to be an important feature of E2F-mediated growth regulation. Overexpression of E2F-1 in serum-starved fibroblasts leads to their entry into S-phase followed by apoptosis (Qin et al., 1994; Shan and Lee, 1994; Kowalik et al., 1995). Furthermore, blocking E2F activity with dominant negative DP1 inhibits apoptosis induced by serum starvation of HBL-100 breast epithelial cells (Bargou et al., 1996). A positive role for E2F-1 in apoptosis in vivo is supported by the increased apoptosis in transgenic megakaryocytes overexpressing E2F-1 (Guy et al., 1996) and by the reduced apoptosis observed in *E2F-1* knockout mice (Field et al., 1996). Although E2F-1 is able to induce p53-independent apoptosis, its apoptotic function is enhanced by p53 and the majority of the apoptosis seen in many cell types is p53-dependent (Wu and Levine, 1994; Kowalik et al., 1995; Hsieh et al., 1997; Phillips et al., 1997). Based on the finding that overexpression of other E2Fs could not induce apoptosis in serum-starved fibroblasts, it has been proposed that induction of apoptosis is a property unique to E2F-1 (DeGregori et al., 1997); however, this finding may be cell type-specific since E2F-2, and to a lesser extent E2F-4, could also induce apoptosis in p16-arrested U343 astrocytoma cells (Dirks et al., 1998). Whether the apoptotic effects of E2F-2 and -4 in these latter cells reflect an independent activity or are due to their ability to induce E2F-1 remains to be determined (Dirks et al., 1998).

As with growth regulation by E2F, E2F-mediated apoptosis is regulated by pocket proteins. pRB can inhibit apoptosis in both p53-positive and p53-negative cells (Qin et al., 1994; Haas-Kogan et al., 1995; Shan et al., 1996a; Hsieh et al., 1997), and loss of pRB is linked to both p53-dependent and -independent apoptotic defects in transgenic animals (Macleod et al., 1996; Zacksenhaus et al., 1996). Since mutations of E2F-1 that inhibit its interaction with pRB also block the ability of pRB to block E2F-1-mediated apoptosis, this effect must be due, at least in part, to direct modulation of E2F function by pRB (Shan et al., 1996a). A direct connection between E2F-1 and pRB in apoptosis is also supported by the finding that loss of E2F-1 is able to significantly suppress abnormal apoptosis observed in *RB* knockout mouse embryos (Tsai et al., 1998). The effects of pRB on apoptosis also appear to be regulated by cyclin-dependent kinases since the relative ability of overexpressed cyclin D1 and cyclin E to induce apoptosis correlated with their ability to cause hyperphosphorylation of pRB in serum-starved cells (Soferlevi and Resnitzky, 1996).

E2F-1 mutants from which the transactivation domain has been deleted are unable to induce apoptosis in REF52 and RAT-2 cells (Shan and Lee, 1994; Krek et al., 1995). However, since the transactivation domains of E2F-1 interact with factors implicated in regulation of both p53-dependent and -independent apoptosis (e.g. p300, Mdm2 and pRB, see below), it is not clear whether the effects of deletion of the E2F-1 transactivation domain are due to loss of transactivation activity or loss of such interactions. Furthermore, E2F transactivation function is not required for apoptosis in all circumstances. Transactivation-defective E2F-1 is able to induce apoptosis in p53-negative SAOS-2 cells (Hsieh et al., 1997). Since the requirement for E2F transactivation domains for induction of apoptosis has been studied in multiple cell lines, the apparently contradictory results may reflect differences in the requirements for induction of p53-independent and p53-dependent apoptosis, although this remains to be proven. The ability of pRB to block E2F-1-mediated apoptosis presumably relies on its masking of the E2F-1 transactivation domain in cells where this domain is required, but pRB-mediated repression (see below) is likely to mediate this anti-apoptotic activity in cells where the E2F-1 transactivation domain is dispensable for induction of apoptosis. Consistent with a role for pRB-mediated repression in regulation of apoptosis, peptides that block E2F-DNA binding and transactivation function can induce apoptosis in several tumor cell lines (Bandara et al., 1997). It should also be noted that factors other than E2F-mediated transcriptional activation, pRB-mediated repression, and interference with p53 apoptotic function appear to be involved in E2F-1-induced apoptosis, based on the ability of transactivation-defective E2F-1 to induce apoptosis in SAOS-2 cells which lack functional pRB in addition to being p53-negative (Hsieh et al., 1997).

The exact mechanisms by which E2F induce apoptosis, and how these relate to its cell cycle regulatory functions, remain to be elucidated. One proposed mechanism is that E2F-1-mediated apoptosis is linked to

enhanced E2F-1 transactivation in S-phase (e.g. Krek et al., 1995); however, other mechanisms must also be involved since results from analysis of cycling Rat1 fibroblasts cells show that E2F-1 overexpression only induced apoptosis in G_1 cells and that S-phase cells are resistant to the apoptotic effects of E2F-1 (Hengstschlager et al., 1999).

Studies of apoptosis-inducing treatments have indicated a role for endogenous E2F-1 in apoptosis. Treatment of HL-60 cells with ionizing radiation leads to upregulation of E2F-1 protein, downregulation of cyclin A-associated kinase activity, and induction of S-phase and apoptosis (Huang et al., 1997). E2F DNA binding and/or protein levels are also upregulated by UV irradiation and antineoplastic drugs (Hofferer et al., 1999; Meng et al., 1999). Direct involvement of E2F in apoptosis induced by these DNA damaging treatments is supported by (a) the ability of dominant negative DP1 to block both the induction of S-phase and apoptosis in irradiated HL-60 cells (Huang et al., 1997), and (b) the increased radiosensitivity of p53-negative fibrosarcoma cells following E2F-1 overexpression (Pruschy et al., 1999). Although induction of E2F-1 levels in response to DNA damage appears to involve mechanisms similar to those for p53 (Blattner et al., 1999), it can occur in a p53-independent manner (Meng et al., 1999). Thus, increased expression of E2F-1 may represent a primary mechanism for induction of apoptosis, at least in some circumstances.

The importance of E2F-1 in apoptosis during normal development is demonstrated by the observed apoptotic consequences of deleting *E2F-1* and *RB* in transgenic mice. Since circumvention of apoptosis is a key aspect of tumorigenesis, the apoptotic functions of E2F are also likely to be deregulated in cancer and therefore represent a potential target for cancer therapy.

7. Repression versus activation of transcription by E2F

Since overexpression of E2Fs upregulates transcription of E2F target genes, initial versions of the pRB·E2F model assumed that E2F was solely an activating factor whose activity was blocked by pocket protein binding. However, increasing evidence from a number of promoters has indicated that E2F sites mediate repression in G_0 as well as activation in G_1. For example, mutation of the E2F site in the *B-myb* promoter leads to loss of cell cycle regulation due to enhanced transcription in G_0 rather than decreased activity in G_1 (Lam and Watson, 1993). Similar results have been found for other promoters such as those for E2F-1, pRB, HsOrc1, and hamster DHFR (Neuman et al., 1994; Ohtani-Fujita et al., 1994; Ohtani et al., 1996; Jensen et al., 1997). Consistent with lack of E2F-mediated activation in G_1, both in vivo footprinting and formaldehyde crosslinking have revealed that E2F binds to the *B-myb* promoter in G_0 but not in late G_1 (Zwicker et al., 1996; Wells and Farnham, personal communication). How this relates to the ability of pocket protein interaction to stabilize the binding of E2F to DNA remains to be determined (Huber et al., 1994).

Although growth/cell cycle-dependent regulation of E2F-dependent promoters through inhibition of E2F-dependent activation or repression of promoter activity in G_0/early G_1 would tend to have the same overall outcome — activation of promoters in mid/late G_1 — mechanisms for these modes of regulation would clearly be different. Inhibition of E2F activation by pocket proteins resides in their masking of the transactivation domain of E2F which interferes with its interaction with the basal transcription machinery (Ross et al., 1999; Pearson and Greenblatt, 1997). Additionally, pocket proteins may alter transactivation through their ability to affect E2F-mediated DNA bending (Huber et al., 1994; Cress and Nevins, 1996). Pocket protein interaction also plays a role in E2F-mediated repression since introduction of pRB into pRB-negative cells can change E2F sites from positive to negative promoter elements (Weintraub et al., 1992). Indeed, E2F mediates repression by recruiting pocket proteins since pocket proteins can directly repress the activity of other transcription factors when recruited to the promoter by E2F or when bound directly to the promoter through fusion to a heterologous DNA binding domain (Adnane et al., 1995; Bremner et al., 1995; Sellers et al., 1995; Chow and Dean, 1996; Starostik et al., 1996; De Luca et al., 1998).

Pocket protein-mediated repression is achieved by at least two mechanisms. One involves the histone deacetylase, HDAC1 (Brehm et al., 1998; De Luca et al., 1998; Ferreira et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998; Stiegler et al., 1998a). Acetylation of histones weakens the association of nucleosomes with DNA which allows for more efficient transcription (Struhl, 1998). Recruitment of a histone deacetylase leads to histone deacetylation and thus represses transcription. All three pocket proteins can bind to HDAC1: the interaction is mediated by an LxCxE-like sequence in HDAC1 and requires an intact pocket domain in the pocket proteins (Ferreira et al., 1998; Magnaghi-Jaulin et al., 1998). E2Fs and HDAC1 can simultaneously bind to pocket proteins (Ferreira et al., 1998), thereby enabling E2F to recruit histone deacetylase to the promoter at times when pocket proteins are hypophosphorylated. Involvement of histone deacetylase activity in E2F-mediated repression is supported by (a) the ability of wild type HDAC1 (but not HDAC1 containing a mutation in its LxCxE-like sequence) to augment pRB-mediated repression of E2F site-containing promoters (Magnaghi-Jaulin et al., 1998), and (b) the ability of

trichostatin A, a histone deacetylase inhibitor, to partially inhibit repression of promoters by pocket proteins that are fused to a heterologous DNA binding domain or recruited by E2F (Brehm et al., 1998; Luo et al., 1998). Furthermore, G_1 phosphorylation of pocket proteins, viral oncoproteins which target the pocket, or oncogenic mutations in the pocket disrupt the interaction of pocket proteins with HDAC1 (Brehm et al., 1998; Ferreira et al., 1998; Magnaghi-Jaulin et al., 1998), indicating that this interaction is important in the growth regulatory function of this class of tumor suppressors.

Another mechanism of pocket protein-mediated repression, which was demonstrated for pRB, differs from that involving HDAC1 recruitment in that it is restricted to certain transcription factors and is insensitive to histone deacetylase inhibition. This repression is seen with transcription factors that interact with pRB, such as PU.1 and NFκB, and appears to lie in the ability of pRB to block interaction of the transcription factor with components of the basal transcription machinery (Weintraub et al., 1995; Luo et al., 1998). The need for pocket protein·transcription factor interaction was highlighted by the inability of pRB to directly repress USF, Sp1 or CTF1, factors for which no pRB interaction was detected (Luo et al., 1998). Although this mode of repression is mediated by interaction of pocket proteins with target transcription factors, it remains E2F-dependent because the interaction is too weak for pRB to directly repress PU.1 or NFκB activity and thus requires pRB to be concentrated at the promoter through its interaction with E2F (or through its fusion to a heterologous DNA binding domain) (Weintraub et al., 1995).

A third mechanism of E2F-mediated promoter repression has come to light with the identification of a cell cycle gene homology region (CHR) in the *B-myb* promoter (Liu et al., 1996). This sequence element is found in a number of cell cycle regulated genes that are induced in S-phase or G_2 such as *cyclin A*, *cdc25* and *cdc2* (Zwicker et al., 1995). However, the sequence in *B-myb* is functionally different from that in the latter promoters in that it is involved in G_1 induction of transcription and appears to bind a different protein complex (Liu et al., 1996). Mutational analysis of the *B-myb* promoter has indicated that repression of this promoter in G_1 requires both an intact E2F site and an intact CHR (Bennett et al., 1996; Liu et al., 1996). Since both E2F and the CHR binding factor can bind to the promoter when the other site is mutated, the exact nature of the interaction between these sites is unknown. Although pocket protein binding to the *B-myb* promoter has been correlated with its repression (Raschella et al., 1998), CHR-dependent repression differs from the pocket protein-dependent repression noted above which is seen with promoters which lack a CHR and is dependent solely on recruitment of pocket proteins to the promoter.

Prediction of whether an E2F site acts as a repressive or an activating promoter element is complicated not only by the ability of pocket proteins to switch this phenotype, but also by the fact that the high, non-physiological levels of E2F obtained following transfection can lead to activation of promoters that have otherwise been shown to be repressed through E2F sites. For example, overexpression of E2F leads to activation of the *B-myb* promoter (Pierce et al., 1998); however, as mentioned above, the E2F sites in this promoter are occupied only during G_0/early G_1 and are repressive rather than activating when E2F is expressed at physiological levels. Similarly, E2F overexpression can activate other promoters where E2F sites are normally repressive, such as HsOrc1 and E2F-1 (Ohtani et al., 1996; Pierce et al., 1998). The repressive and activating nature of E2F sites can be changed by altering their positioning within a promoter or by changing the identity of the other transcription factors that bind to the promoter (Van Ginkel et al., 1996; Fry et al., 1997). Indeed, differences between E2F sites can be seen in a single promoter: both the p107 and *RanBP1* promoters contain two E2F sites which differ in their ability to mediate repression and activation (Zhu et al., 1995; Di Fiore et al., 1999). Since mouse strains have been shown to differ with regard to induction of E2F-1 during liver regeneration and in the consequences of *p107* knockout (Bennett et al., 1995; LeCouter et al., 1998), cell type and strain differences are also likely to affect the repressive/activating potential of E2F sites. In this regard, it is noteworthy that E2F sites of the hamster DHFR promoter are activating in HeLa cells (which lack pRB function) but are repressive in Balb/c 3T3 cells (Blake and Azizkhan, 1989; Jensen et al., 1997), and the E2F sites of the closely related mouse DHFR promoter are both repressive and activating in NIH 3T3 cells (Fry et al., 1997). Therefore, E2F appears to play a bipartite role in regulation of transcription — being a negative factor in G_0 and activating transcription in G_1, with the exact effect dependent on the promoter and cell type.

Studies on cell growth in the absence of E2F transactivation activity have highlighted the importance of E2F-mediated repression. *E2F-1* knockout mice display hyperproliferation in the thymus and have increased incidence of tumors, indicating that E2F-1 can act to inhibit cell growth and has properties of a tumor suppressor (Field et al., 1996; Yamasaki et al., 1996). Abrogation of E2F transactivational activity by dominant negative proteins did not alter the cell cycle profile of rat embryo fibroblasts or HBL-100 cells but did increase their tumorigenicity (Jooss et al., 1995; Bargou et al., 1996). Dominant negative, transactivation domain deficient E2F can also block growth arrest induced by

$p16^{ink4a}$ in U2OS cells, and by TGFβ or contact inhibition in mink lung epithelial cells, without affecting the cell cycle profiles of growing cells (Zhang et al., 1999b). Similarly, although mutation of the stimulatory E2F in *Drosophila* is lethal, loss of E2F transactivation does not necessarily prevent cell growth/cell cycle progression since mutant flies can develop to the late larval/pupal stage (Royzman et al., 1997; Sawado et al., 1998). Collectively, these studies indicate that E2F transactivation activity is not required for cell cycle progression, suggesting that mediation of repression may be a major role of E2F in the cell. This idea is supported by the finding that dissociation of nuclear E2F·pocket protein complexes resulted in little increase in 'free' nuclear E2F (Verona et al., 1997). The observed tumorigenic and growth effects of overexpressed E2Fs may need to be reconsidered in light of the potential ability of overexpressed E2F to relieve E2F-mediated repression through altered phosphorylation or sequestration of pocket proteins. However, it should also be noted that E2F-dependent activation of transcription clearly has a role in growth/cell cycle regulation since blocking the activity of E2F with E2F oligonucleotides or with dominant negative proteins can block the ability of cells to enter S-phase (e.g. Dobrowolski et al., 1994; Wu et al., 1996; Akimoto et al., 1998; Maeshima et al., 1998). Furthermore, the evolutionary conservation of transactivation function in E2F family members indicates that activation of transcription is an important role of E2F; however, the precise nature of this role remains to be elucidated.

8. Growth versus cell cycle regulation by E2F

Although the role of E2F in transcriptional regulation following serum induction of quiescent cells (i.e. growth stimulation) is well established, very few studies have addressed the role of E2F in regulation of gene expression in the ongoing cell cycle of actively growing cells. This is an important consideration since there is evidence that the function of E2Fs during re-entry into the cell cycle is different from that in cycling cells. Cells in which regulation of E2Fs is disrupted by viral oncoprotein-mediated abrogation of pocket protein interaction are refractory to growth arrest but still progress through the cell cycle. Studies using fibroblasts from *E2F-1* knockout mice indicate that E2F-1 deficiency delays re-entry into the cell cycle from quiescence but does not affect the cell cycle in actively growing cells (Wang et al., 1998). However, it is likely that E2F does regulate transcription during the ongoing cell cycle since the localization of E2Fs and E2F·pocket protein complexes varies with the cell cycle in actively dividing cells (Verona et al., 1997) and inactivation of E2F activity using dominant negative DP1 or through the use of decoy oligonucleotides can prevent entry of growing cells into S-phase both in vitro and in vivo (e.g. Morishita et al., 1995; Wu et al., 1996; Fan and Bertino, 1997; Akimoto et al., 1998; Tomita et al., 1998).

At the level of gene expression, differences in expression of E2F-responsive genes during an ongoing cell cycle have been reported [although it should be noted that most of these studies have concentrated on protein or RNA levels, with few (e.g. Farnham and Schimke, 1985; Leone et al., 1998) directly assessing transcription]. Studies comparing RNA levels in REF52 cells following release from serum starvation and hydroxyurea block indicated that expression of cyclin E, cdc6, cdk2 and PCNA were similarly regulated following cell cycle re-entry and during an ongoing cell cycle (Leone et al., 1998). However, this is not true for all such genes since RNA levels for E2F-1 to -3 and thymidine kinase were growth-regulated but did not vary in cycling cells. Interestingly, studies on the same gene can yield different results; for example, expression of thymidine kinase RNA, which was not regulated in cycling REF52 cells (Leone et al., 1998), was found to be cell cycle-regulated following centrifugal elutriation of actively growing primary human and mouse fibroblasts (but not in cells transformed with viral oncogenes that disrupt E2F regulation) (Hengstschlager et al., 1994). From these studies, it is clear that E2F-regulated, growth-responsive genes differ with respect to regulation of their expression during an ongoing cell cycle (and that these differences may be cell type-specific), indicating that there is a level(s) of regulation not apparent from studies of serum stimulation of quiescent cells. Therefore, although not a trivial undertaking, a complete understanding of cell cycle regulation of transcription by E2F will require further studies specifically designed to delineate the role of E2F and E2F sites in cycling cells.

9. Expression of E2Fs during the cell cycle and development

The changes in expression of individual E2F family members differs following serum stimulation of quiescent cells. E2F-4 and -5 mRNA and protein are expressed in quiescent cells and at relatively constant levels throughout the cell cycle, although their expression may increase slightly in mid G_1 (Ginsberg et al., 1994; Sardet et al., 1995; Moberg et al., 1996; Sears et al., 1997). In contrast, E2F-1, -2 and -3 mRNA and protein are undetectable in quiescent cells but are induced in mid–late G_1 (Kaelin et al., 1992; Slansky et al., 1993; Moberg et al., 1996; Sears et al., 1997; Leone et al., 1998). Although regulation of E2F expression during the ongoing cell cycle may be regulated by mechanisms different from those seen in serum-stimulated cells, it appears to follow a similar pattern since E2F-1 and -3

levels are upregulated in late G_1/S and E2F-4 is found throughout the cell cycle (see E2F localization and Proteolysis sections). Consistent with their role as partners for all E2Fs, expression of DP1 and DP2 does not vary significantly during the cell cycle (Bandara et al., 1994).

Pocket protein expression is also cell cycle-regulated. p130 is the major pocket protein in G_0 in many cell types (Moberg et al., 1996; Smith et al., 1996; Williams et al., 1997; Smith et al., 1998). However, following serum stimulation, p130 levels drop due to proteasome-mediated degradation and expression of p107, and to some extent pRB, is induced in late G_1 (Smith et al., 1998).

In keeping with this regulation of expression, different E2F·pocket protein complexes are observed at different stages of the cell cycle. p130 complexes are the major complexes found in G_0 in many cell types, with pRb and p107 complexes being prominent only following re-entry into the cell cycle and in cycling cells (Moberg et al., 1996; Smith et al., 1996; Williams et al., 1997). Thus, it has been proposed that p130 is primarily involved in growth inhibition in quiescent cells and that pRB and p107 are involved in regulation of actively growing cells. This idea is supported by the expression of pocket proteins during renewal of the intestinal epithelium in vivo. pRB and p107 are highly expressed in proliferating cells of the intestinal crypt and are markedly downregulated in early differentiating cells at the crypt–villus junction. In contrast, p130 is weakly expressed in proliferating cells of the lower crypt, but is induced at the exact point within the crypt where cells stop dividing (J.D. Frey and M. Black, in preparation). However, a role for pRB in non-growing cells is suggested by (a) the further downregulation of its expression as intestinal epithelial cells become fully mature on the villus (Frey and Black, personal communication), (b) the presence of E2F·pRB complexes in quiescent primary human B-lymphocytes and differentiated HL60 cells (Ikeda et al., 1996; van der Sman et al., 1999), and (c) the defects in differentiation and cell cycle exit in neuronal and muscle cells lacking pRB (Novitch et al., 1996; Callaghan et al., 1999).

Consistent with findings in cultured cells, studies of E2Fs during development of many species show a general correlation of E2F expression with cell growth (Pasteau et al., 1995; Rubin et al., 1998). Taking mouse as an example, in skin and gut epithelium, expression of E2F-2 and E2F-4 is seen in undifferentiated cells, with E2F-5 only appearing following the onset of stratification or the development of differentiated cell types in these tissues, respectively (Dagnino et al., 1997b). However, expression in some tissues does not fit with the in vitro model. For example, in late gestation, E2F-1 is expressed in non-dividing brown fat of the neck and in non-dividing cells of the uterus (Tevosian et al., 1996). In the retina, while E2F-1 expression is restricted to non-differentiated, dividing cells, E2F-3 is only expressed in differentiated ganglion layer cells (Dagnino et al., 1997a).

Some findings indicate that E2F may not be critical for expression of growth-related genes during early development in some tissues; for example, transcripts for E2F-1 to -5 were undetectable or barely detectable in the mouse intestine prior to 12.5 days of gestation (Dagnino et al., 1997b). This idea is supported by the finding that *Drosophila* bearing mutants in the sole activating E2F gene or DP genes can develop to the late larval/pupal stage despite a loss of E2F-dependent transcription (Royzman et al., 1997). Interestingly, transgenic mouse studies indicated that E2F-5 plays a role in post-proliferative choroid plexus, indicating that E2Fs can have roles unrelated to control of growth and apoptosis (Lindeman et al., 1998).

Expression of DP1 during development is also consistent with the levels and general expression of DP1 in tissue culture cells since DP1 expression was observed in most tissues during development at levels equivalent to or higher than individual E2Fs (Tevosian et al., 1996; Dagnino et al., 1997a; Loiseau et al., 1997). However, as with E2Fs, levels of DP1 were low in the early mouse embryo and some tissues, such as the choroid plexus, did not express DP1 at any stage of development. Also consistent with data from cell lines, DP2 expression appears to be more tissue-restricted than DP1 (Zhang and Chellappan, 1995).

Although the expression of E2F and DP proteins during development has generally supported the roles of E2F discerned in vitro, the data also indicate that the roles of E2F are more complicated than predicted from in vitro models. Therefore, full elucidation of the functions of this transcription factor family in growth regulation will require careful comparison of results obtained in vitro with those obtained in vivo. For these studies, systems such as that developed for the intestinal epithelium, that allows direct comparison of growth regulatory events in the IEC-18 cell culture model with those occurring in vivo, should prove highly useful (Saxon et al., 1994; Frey et al., 1997).

10. Interaction of E2Fs with other factors

In addition to the interaction of E2Fs with pocket proteins and cyclins, interaction with other cellular proteins impacts on the functions of E2F in cell growth control and transcription.

10.1. Sp1

Many growth-regulated genes contain binding sites for both E2F and Sp1, implying that this combination may be relevant to the regulation of such genes. Indeed, analysis of the *c-myc*, *DHFR* and mouse *thymidine*

kinase promoters has revealed that the E2F and Sp1 sites functionally interact to cooperatively activate transcription (Majello et al., 1995; Karlseder et al., 1996; Lin et al., 1996). This functional interaction appears to be mediated by a physical interaction between these two transcription factors (Karlseder et al., 1996; Lin et al., 1996). Interestingly, Sp1 directly interacts with E2F-1, -2 and -3, whose expression is growth-regulated, but not with the more constitutively expressed E2F-4 and -5. Therefore, this interaction may enable E2F to exert cell cycle/growth regulation of transcription on promoters that lack classical E2F sites. In this regard, it is significant that following serum stimulation of quiescent Balb/c 3T3 cells, Sp1 sites can mediate induction of transcription from the hamster DHFR promoter in late G_1 (Jensen et al., 1997), i.e. at the time when E2F-1 to -3 are induced. Although it remains to be determined whether this Sp1-site-mediated activation is dependent upon the interaction of E2F with Sp1, the induction of DHFR transcription correlates with changes in Sp1 phosphorylation within the region where it interacts with E2F but not with changes in Sp1 levels (Black et al., 1999). Interestingly, Sp1 is also regulated in a positive manner by pRB (Kim et al., 1992; Udvadia et al., 1995), adding a further level of complexity to the regulation of promoters containing these two factors.

10.2. CBP/p300

CBP and p300 are closely related transcriptional co-activators which can stimulate the activity of a wide range of transcription factors (see Goldman et al., 1997; Snowden and Perkins, 1998 for a review). Both CBP and p300 bind to E2F-1 through a site in the transactivation domain of E2F-1 (Trouche et al., 1996; Lee et al., 1998). Co-expression experiments revealed that CBP or p300 can enhance E2F-1 transcriptional activity. In keeping with this observation, point mutations of E2F that abolish its CBP binding lead to an eight-fold reduction in transactivation activity of E2F-1 in U2OS cells (Trouche et al., 1996). The role of CBP/p300 binding in E2F activation is further supported by studies using the mouse DHFR promoter indicating that the transactivating ability of E2F-1 mutants correlates with their ability to bind to CBP, rather than their binding to TFIIH or TBP (Fry et al., 1999).

CBP and p300 each contain a histone acetylase function and can also recruit the histone acetylase, PCAF. In this regard, their interaction with E2F-1 offers an interesting contrast with the E2F·pRB interaction which recruits histone deacetylase (see above). The involvement of CBP/p300 binding with E2F transactivation indicates that recruitment of these acetylases following release of E2F-1 from pRB may represent an important mechanism in the relief of pRB-mediated transcriptional repression.

10.3. p53 and p53-associated proteins

The ability of overexpressed E2F-1 to induce p53-dependent apoptosis indicates that E2F and p53 regulatory pathways intersect. In addition to the ability of p53 to regulate E2F through induction of $p21^{WAF1/CIP1}$ and subsequent inhibition of pocket protein phosphorylation, these proteins interact at multiple other levels. E2F·DP1 heterodimers interact with p53 directly and this interaction can inhibit the transactivation function of both E2F and p53 (O'Connor et al., 1995; Sorensen et al., 1996). Inhibition is mediated, at least in part, through interaction of the C-terminus of DP1 with the N-terminus of p53 (Sorensen et al., 1996). This interaction enables p53 to compete with E2F-1 for DP1 and thereby inhibits E2F DNA binding. Interestingly, p53 interacts with an immunologically distinct form of DP1 which has been shown to be phosphorylated and cell cycle-regulated (Bandara et al., 1994; Sorensen et al., 1996). p53 can also bind directly to E2F-1 and this interaction also appears to downregulate the activity of both factors (O'Connor et al., 1995). The functional interaction of E2F-1 and p53 is complicated by the association of E2F-1 with other p53-associated proteins. Both E2F-1 and p53 interact with p300, which enhances the transcriptional activity of both factors (Lee et al., 1998). Furthermore, p300 has opposing effects on the apoptotic function of these two proteins; while the p53-independent apoptotic activity of E2F-1 is enhanced by p300, the apoptotic function of p53 is inhibited by the co-activator. Therefore, the interaction of E2F-1 with p300 has been proposed to be involved in the ability of E2F to induce p53-dependent apoptotics, since E2F-1 can compete with p53 for p300, thereby increasing the apoptotic activity of p53 (Lee et al., 1998). p53 is regulated by Mdm2 which targets p53 for protosome-mediated degradation through a mechanism that appears to involve p300 (Giaccia and Kastan, 1998; Grossman et al., 1998). E2F-1 also binds to Mdm2, which can enhance E2F activity in pRB-positive U2OS cells (Martin et al., 1995). However, the precise mechanism of this enhancement requires clarification since the interaction domain for Mdm2 is very close to the pRB binding domain and it has not been demonstrated that Mdm2 does not displace pRB. The binding of E2F-1 to Mdm2 indicates that it could sequester this negative regulator of p53 and, indeed, E2F-1 overexpression leads to an accumulation of p53, which in turn can be blocked by Mdm2 overexpression (Kowalik et al., 1998). In keeping with this interaction, Mdm2 is able to block the ability of E2F-1 to induce p53-dependent, but not p53-independent, apoptosis (Hsieh et al., 1997; Kowalik et al., 1998). E2F is also able to influence Mdm2 function through its ability to induce $p19^{ARF}$, a protein that also binds Mdm2 and stabilizes p53 (DeGregori et al., 1997; Stott et al., 1998).

Mdm2 can also bind to pRb and downregulate its growth suppressive function (Xiao et al., 1995). This interaction leads to a complex relationship between E2F-1, p53 and pRB in apoptotic signaling. pRB can inhibit E2F-1-mediated apoptosis (Qin et al., 1994); however, through its interaction with Mdm2, pRB can stabilize p53 and enhance p53-dependent apoptosis (Hsieh et al., 1999). Therefore, the interplay between p53, pRB and E2F is highly complex; whether signals that induce E2F activity lead to cell growth or apoptosis will depend on a fine balance of the effects on individual members of this pathway.

10.4. Interaction with other factors

Interactions of E2F with an increasing number of factors that regulate growth in a variety of tissues are being identified. The oncogenic viral tyrosine kinase, v-abl, is able to interact with E2F-1 and can stimulate E2F-1-mediated transcription in an interaction-dependent manner (Birchenall-Roberts et al., 1997). v-abl can bind to either the DNA binding or transactivation domain of E2F-1 through its kinase domain. Since the kinase domain is conserved in c-abl, E2F-1 is also likely to interact with this cellular proto-oncoprotein. Indeed, c-abl can stimulate E2F-1-mediated transcription. c-abl also binds to pRB (Welch and Wang, 1993), indicating that c-abl-mediated signal transduction interacts with E2F regulation at multiple levels. Intersection of interferon signaling and E2F regulation is seen with p202, an interferon-inducible protein which inhibits growth. p202 binds E2F-1·DP1 and E2F-4·DP1 and can block E2F-dependent transactivation by blocking DNA binding. However, since p202 can bind to E2F-4·pocket protein complexes, it may be involved in derepression of promoters in addition to inhibition of transactivation. The precise roles of p202 are further complicated by its interaction with many other growth-related factors including pRB, AP1, c-fos, c-jun and NFκB (Choubey et al., 1996; Choubey and Gutterman, 1997). Both E2F-1 and E2F-4 interact with the tumor suppressor BRCA1 in vitro; however, this interaction requires further characterization since BRCA1 only appears to bind to E2F-4 in vivo (Cui et al., 1998). NPDC-1, a protein involved in control of neuronal proliferation and differentiation, interacts with E2F-1. Interestingly, expression of NPDC-1 in adult brain correlates with E2F-1 mRNA levels, suggesting a role for this protein in regulation of this E2F family member (Dupont et al., 1998). Interaction of E2F with cyclin·cdk complexes and other kinases will be discussed below in regard to its phosphorylation.

Direct searches for E2F-interacting proteins have also identified novel factors that can modulate E2F activity. DIP, a BTB/POZ domain protein related to the *Drosophila germ cell-less* gene, binds to DP proteins, localizes them to the nuclear periphery, and inhibits growth and E2F-dependent transactivation in a DP interaction-dependent manner. DIP is expressed widely, with high levels in spermatogenesis, and has a DNA binding-dependent repressive function in its own right; however, its functions remain to be elucidated (de la Luna et al., 1999). E2FBP1, a helix–loop–helix protein that lacks the basic region and leucine zipper, binds to both E2F-1 and DP1; however, only E2F·E2FBP1 heterodimers bind DNA. E2FBP1 enhances E2F-dependent transcription but only in a narrow ratio of E2F to E2FBP1 (Suzuki et al., 1998). Again the function of this protein is unknown.

The interaction of E2F with many growth-regulatory proteins highlights its importance in regulation of cellular proliferation. Clearly, these interactions allow for additional levels of control of E2F, especially since several of these proteins also interact with each other. At present, these interactions are best characterized for E2F-1 and it will be interesting to determine which interactions show specificity among E2F family members, such as that seen with Sp1. Elucidation of the roles of these interactions will also require characterization of how they are regulated and the impact of this regulation on E2F-dependent transcription.

11. Phosphorylation of E2Fs

In addition to regulation of E2F activity through phosphorylation of pocket proteins, direct phosphorylation of E2F also influences its activity. E2F-1 binds to and is phosphorylated by cyclin A·cdk complexes (Mudryj et al., 1991; Dynlacht et al., 1994b; Xu et al., 1994). This interaction is essential for phosphorylation of E2F by cyclin A·cdk since E2F family members and mutant E2F-1 proteins lacking the cyclin A binding domain are not phosphorylated by these complexes (Adams et al., 1996; Dynlacht et al., 1997). Both cyclin A·cdk2 and cyclin A·cdc2 can phosphorylate E2F-1 on multiple sites. One of these sites has been mapped to serine 375, phosphorylation of which enhances E2F binding to pRB (Peeper et al., 1995). In vitro, cyclin A·cdc2 phosphorylates this site more efficiently than cyclin A·cdk2; therefore, this phosphorylation has been proposed to contribute to downregulation of E2F activity in late S/G_2 phase. Cyclin A·cdk2 and cyclin A·cdc2 phosphorylation also leads to downregulation of E2F transactivation activity by reducing the binding of E2F·DP1 heterodimers to DNA (Dynlacht et al., 1994b; Krek et al., 1994). Consistent with this role of cyclin A, E2F DNA binding is downregulated in S-phase following serum stimulation of quiescent NIH 3T3 cells (Krek et al., 1994). Cyclin A·cdk bound to E2F can phosphorylate DP1 in its N-terminal region (Krek et al., 1995) and this phosphorylation has been correlated with

downregulation of E2F DNA binding (Krek et al., 1994). Physiological effects of the phosphorylation of DP1 were demonstrated by the S-phase arrest induced in E2F-1 overexpressing cells by a DP1 mutant lacking all relevant potential phosphorylation sites (Krek et al., 1995). However, the precise role of DP1 phosphorylation remains to be elucidated since (a) phosphorylation-defective DP1 did not arrest cells in the absence of E2F-1 overexpression, and (b) phosphorylation of DP1 in serum-stimulated NIH 3T3 cells correlated with the increase in E2F binding in late G_1 (Bandara et al., 1994). Cyclin A·cdk2 can also downregulate E2F DNA binding activity by direct phosphorylation of E2F-1 since it can inhibit the binding of purified, bacterially expressed E2F-1 in a phosphorylation-dependent manner (Kitagawa et al., 1995). Whether this downregulation is through phosphorylation of serine 375 or through another site on E2F-1, perhaps at an N-terminal site analogous to those in DP1, remains to be determined.

E2F-1 is also phosphorylated on serines 332 and 337 (Fagan et al., 1994). This phosphorylation has effects distinct from cyclin A-mediated phosphorylation in that it inhibits pRB binding to E2F-1 and, therefore, would be involved in activation of E2F-site containing promoters. Consistent with this role, phosphopeptide mapping of serum-stimulated NIH 3T3 cells indicates that this phosphorylation occurs in late G_1 (Fagan et al., 1994). The kinase(s) involved in this phosphorylation in vivo remains to be determined. Cyclin B·cdc2 can phosphorylate E2F-1 on these sites in vitro (Fagan et al., 1994); however, the expression of cyclin B in G_2/M when E2F·pRB complexes reform indicates that, if such phosphorylation occurs in vivo, it is likely to be involved in modulating E2F stability rather than in activation of transcription, since interaction with pocket proteins also inhibits degradation of E2Fs (see below). Phosphorylation of serines 332 and 337 in late G_1 suggests cyclin D·cdk4 or cyclin E·cdk2 as candidate kinases. However, published in vitro phosphorylation data indicate that E2F-1 is not a substrate for cyclin D·cdk4 and is at best a poor substrate for cyclin E·cdk2 (Dynlacht et al., 1994b; Kitagawa et al., 1995; Dynlacht et al., 1997).

Consistent with the multiple phosphorylations of E2F-1, comparison of phosphopeptide maps of in vitro and in vivo labeled E2F-1 indicates that the protein is phosphorylated by kinases other than cyclin A·cdk and cyclin B·cdk (Fagan et al., 1994; Kitagawa et al., 1995). The complete inhibition of E2F-1 phosphorylation by the cdk inhibitor butyrolactone 1 in IMR32 neuroblastoma cells argues that cyclin-dependent kinases are significant kinases of E2F-1. In this regard, it is interesting that cdk3 can bind to E2F-1·DP1 complexes through DP1 and appears to be involved in activation of E2F-1 in late G_1 through a mechanism which does not involve pRB or modulation of E2F DNA binding activity (Hofmann and Livingston, 1996). However, the recent finding that JNK can bind to DP1 and can phosphorylate E2F-1 in vitro demonstrates that other kinases can play a role in E2F regulation. Phosphorylation of E2F by JNK results in a reduction in E2F-1 DNA binding which correlates with reduced cellular E2F activity following JNK activation (Wang et al., 1999). Thus, in keeping with its central role in growth control, E2F is likely to be a focal point of multiple signaling pathways that affect growth and cell cycle-regulated transcription.

Little information is available concerning the role of phosphorylation of other E2F family members, although DP2, E2F-4 and E2F-5 have also been shown to be phosphoproteins (e.g. Ginsberg et al., 1994; Rogers et al., 1996; Vaishnav et al., 1998). E2F-2 and -3 are highly homologous to E2F-1 and share the cyclin A binding domain and contain a serine equivalent to amino acid 375 of E2F-1. Furthermore, based on studies utilizing dominant negative mutants, cdk3 appears to regulate E2F-1, -2 and -3 similarly (Hofmann and Livingston, 1996); therefore, these three proteins are likely to share aspects of regulation by phosphorylation. However, differences must also exist since E2F-2 and -3 lack amino acids analogous to serines 332 and 337 of E2F-1 (Fagan et al., 1994). E2F-4 and E2F-5 lack a cyclin A binding domain and E2F-4 has been demonstrated not to be directly regulated by cyclin A·cdk complexes (Dynlacht, 1997). Although cyclin A can interact indirectly with E2F-4 and -5 through p107 and p130, these complexes are of unknown function (see above). In contrast to E2F-1·DP1, phosphorylation of E2F-4·DP1 complexes appears to enhance their DNA binding since dephosphorylation of these complexes correlates with reduced DNA binding in S-phase human primary B-lymphocytes (van der Sman et al., 1999). Differences between the regulation of E2F-4/5 and E2F-1 to -3 are highlighted by the apparent lack of effect of dominant negative cdk3 on E2F-4 activity (Hofmann and Livingston, 1996).

A number of studies have investigated the physiological relevance of E2F phosphorylation. When compared with wild type protein, overexpression of E2F-1 lacking its cyclin A binding sites, and therefore defective in cyclin A·cdk phosphorylation, leads to enhanced DNA binding by E2F in S-phase, enhanced transcription of S-phase genes, a block in cell cycle progression in S-phase, and to apoptosis (Krek et al., 1995; Hsieh et al., 1997). Similarly, peptides that disrupt interaction of cyclin A with its substrates induce an S-phase arrest and apoptosis in transformed cells and cells overexpressing E2F-1 (Chen et al., 1999). Since these effects required an intact DNA binding domain on E2F-1, phosphorylation-mediated downregulation of E2F-1 binding to promoters is likely to be a key factor in regulation of an S-phase check point (Krek et al., 1995). Regulation of

E2F binding by phosphorylation may be particularly relevant in cycling cells since it has been reported that E2F-1 DNA binding is suppressed during all phases of an ongoing cell cycle in REF52 cells (Leone et al., 1998).

The importance of E2F phosphorylation is also highlighted by its targeting by viral proteins and growth regulatory factors. Adenovirus E1a disrupts the association of cyclin A with E2F-1 (Mudryj et al., 1991). Direct physical interaction and phosphorylation of E2Fs by viral proteins is seen in the case of HCMV IE72 which phosphorylates E2F-1, -2 and -3 and activates E2F-dependent transcription in a manner correlated with its kinase activity (Pajovic et al., 1997). Regulation of E2F-1 phosphorylation also appears to be a mechanism involved in the control of growth by $p21^{WAF1/CIP1}$. Overexpression of $p21^{WAF1/CIP1}$ in p53/pRB negative SAOS-2 cells leads to inhibition of cyclin A·cdk activity and inhibits phosphorylation of E2F-1, with a concomitant increase in E2F binding, and leads to an S-phase/G_2 cell cycle block and increased activity of E2F-responsive genes (Li et al., 1997). In pRB positive cells, such inhibition of E2F phosphorylation by $p21^{WAF1/CIP1}$ may increase growth arrest by enhancing the ability of E2F to recruit repressive pocket proteins to promoters. $p21^{WAF1/CIP1}$ also appears to affect the activity of E2F-1·cyclin A·cdk complexes by additional mechanisms, since induction of $p21^{WAF1/CIP1}$ in BT-20 breast cancer cells by vitamin E succinate correlated with cyclin A binding region-dependent repression of E2F transactivation activity which was not related to changes in E2F DNA binding (Turley et al., 1997). Vitamin E succinate led to decreased cdk2 association and increased $p21^{WAF1/CIP1}$ association with E2F-1, indicating that cyclin A·$p21^{WAF1/CIP1}$ complexes may be able to directly affect E2F transcriptional activity.

Physiologically relevant changes in E2F phosphorylation can also be mediated by changes in phosphatase levels since PPARγ-induced cell cycle withdrawal is linked to downregulation of phosphatase PP2A which correlates with increased phosphorylation of DP1 and decreased E2F activity (Altiok et al., 1997).

Although the regulation of E2F by direct phosphorylation is only beginning to be elucidated, the multiple mechanisms of phosphorylation together with differences in regulation of individual E2F family members offers a complex mechanism to precisely regulate the functions of these proteins during the cell cycle. In addition, this level of regulation of E2Fs forms a complex interlinked network with those involved in pocket protein phosphorylation. Both protein families are regulated by cdks; however, different cdks are involved and phosphorylation can have different net effects on the transcription of E2F site-containing genes. Further potential for differential regulation also exists at the level of phosphatases, where pRB dephosphorylation is linked to PP1 (Rubin et al., 1998) and E2F has been linked to PP2A (Altiok et al., 1997). Therefore, the variety of pathways and the multiple levels of regulation through phosphorylation provide an elegant network for modulation of E2F activity.

12. E2F localization

The subcellular localization of several E2Fs is regulated in a cell cycle-dependent manner (de la Luna et al., 1996; Magae et al., 1996; Allen et al., 1997; Lindeman et al., 1997; Muller et al., 1997; Verona et al., 1997). Whereas E2F-1, -2 and -3 appear to be exclusively nuclear, a significant portion of E2F-4 and -5 is present in the cytoplasm and their localization is cell cycle-regulated in both serum-stimulated quiescent cells and actively cycling cells. In G_0 and early G_1, E2F-4 and -5 are found in the nucleus as well as in the cytoplasm; however, at other stages of the cell cycle they appear to be exclusively cytoplasmic. The differences in the nuclear localization of E2F-1 to -3 and E2F-4/5 are explained by the presence of a nuclear localization signal in the N-terminus of the former but not the latter. The precise mechanism underlying the shift in localization of E2F-4 and -5 remains to be determined. Co-expression with p107 and p130, but not pRB, enhances nuclear localization of E2F-4 and/or -5 in CHO and U2OS cells, suggesting that association with p107 and p130 may be responsible for the redistribution. However, other factors must be involved since analysis of cycling HL60 cells revealed that the majority of E2F·p107 and E2F·p130 complexes were in the cytoplasm, whereas E2F·pRB complexes were exclusively nuclear (Verona et al., 1997). Furthermore, co-expression of E1a did not affect the nuclear localization of E2F-5·DP1 expressed in conjunction with p107, even though it was expressed at levels sufficient to disrupt the nuclear E2F·p107 complexes.

DP proteins also differ in their ability to localize to the nucleus. DP1 lacks a nuclear localization signal and requires association with E2Fs to reach the nucleus (Magae et al., 1999). The ability of DP2 to localize to the nucleus is affected by its alternate splicing, with only some splice variants containing a specific region at the N-terminus which is able to direct nuclear localization (de la Luna et al., 1996). Overexpression of the DP2 splice variants containing the nuclear localization signal leads to compartmentalization of co-expressed E2F-4 to the nucleus, indicating that modulation of DP2 splicing may be a mechanism in the regulation of E2F localization. However, DP2 expression is restricted and E2F-4·DP1 complexes are found in the nucleus even when pocket protein complexes are disrupted by E1a, again indicating that other mechanisms must be involved. The situation with DP1 is more complex since, consistent with its lack of nuclear localization signal, DP1 does not promote nuclear localization of E2F-4/5 in a wide

variety of cell types; however, a significant portion of E2F-4 and -5 was located in the nucleus in REF52 cells when overexpressed with DP1 (DeGregori et al., 1997).

The finding that the majority of cellular E2F activity is due to E2F-4 and that it is localized in the cytoplasm will necessitate reinterpretation of some data concerning the role of this family of transcription factors. For example, although upregulation of E2F-regulated genes correlates with increased levels of 'free' E2F-4 in late G_1 cells (e.g. Wells et al., 1997), this 'free' E2F-4 is likely to be cytoplasmic and, therefore, would not directly participate in transcription. Thus, the coincident rise in expression and 'free' E2F-4 levels may reflect the loss of repressive complexes from the nucleus during the G_0 to S-phase transition, rather than active upregulation of transcription by E2F-4 in G_1. Subcellular localization must also be considered in interpretation of experiments involving overexpression of E2Fs, since the ability to enter the nucleus has been directly correlated with the activity of transfected E2Fs. In contrast to E2F-1, E2F-4 is unable to enter the nucleus and fails to significantly activate transcription or induce S-phase when overexpressed in U2OS or C-33A cells. However, directing E2F-4 to the nucleus of these cells through its fusion to the nuclear localization signal of E2F-1/2 or SV40 large T antigen, or by co-expression with DP2, leads to a significant increase in its transactivational activity and its ability to drive cells into S-phase (Muller et al., 1997; Verona et al., 1997).

The cell cycle regulation of the localization of E2F-4 and -5 would suggest that these family members are mainly involved in repression of transcription in G_0 and early G_1 rather than activation in G_1. Therefore, differences in the regulation of their subcellular localization could lead to significant differences in the activation/repressive activity of E2F sites in different cell lines. Indeed, since E2F-4 represents the majority of the total E2F in the cell, even small changes in the ability of E2F-4 to localize to the nucleus could have profound effects on E2F transcriptional activity.

Regulation of E2Fs by subcellular localization allows for a regulatory system complementary to those discussed above. For example, promoter interaction of all E2Fs appears to be inhibited in S-phase: in the case of E2F-1, -2, and -3, this is through phosphorylation by cyclin A·cdk, whereas for E2F-4 and -5, this is through sequestration in the cytoplasm.

13. Proteolysis

Analysis of the relative expression of transfected E2Fs revealed that both E2F-1 and E2F-4 are unstable proteins which are targeted for degradation by the proteasome in a wide variety of cell types (Hateboer et al., 1996; Hofmann et al., 1996; Campanero and Flemington, 1997; Martelli and Livingston, 1999). These proteins are targeted for proteasome-mediated degradation by sequences near their C-terminus (Hateboer et al., 1996; Hofmann et al., 1996; Campanero and Flemington, 1997). Consistent with the involvement of the C-terminus in targeting E2F for degradation, pocket protein binding stabilizes both E2F-1 and -4, altering their half life from ~ 2 h to > 8 h. Stabilization by pocket proteins appears to be directly linked to their ability to bind E2Fs and mask the destabilization domain, rather than non-specific or cell cycle effects, since (a) mutations in the pocket proteins or E2F-1/4 that preclude their interaction block stabilization of these E2Fs, (b) p107 does not alter the expression of other unstable nuclear proteins, (c) levels of pocket proteins which are unable to cause cell cycle arrest can stabilize E2Fs, (d) $p27^{KIP1}$-induced cell cycle arrest does not stabilize either E2F-1 or -4, and (e) stability was independent of transactivation, DNA binding or cyclin A binding activities of E2Fs (Hateboer et al., 1996; Hofmann et al., 1996). Consistent with a role of pocket proteins in stabilization of E2Fs, their stability appears to be regulated during the cell cycle in REF52 cells and IMR90 human diploid fibroblasts (Leone et al., 1998; Martelli and Livingston, 1999). However, other mechanisms of stabilization are present in the cell, since co-expression of adenovirus E1a and E1b proteins also leads to stabilization of E2F-1 and -4 (Hateboer et al., 1996; Hofmann et al., 1996), and E2F-3 is more stable in S-phase than in G_0 and G_1 in BALB/c 3T3 cells (Flores et al., 1998). One such mechanism may lie in the interaction of E2F-1 with Mdm2, which appears to enhance the degradation of E2F in a manner analogous to its actions on p53 (Blattner et al., 1999).

Although the role of degradation of E2F in its activity remains to be elucidated fully, it appears to be a major level of regulation in cycling cells and during differentiation. Levels of E2F-1 and -3 protein are cell cycle-regulated in REF52 cells in the absence of changes in their mRNA levels (Leone et al., 1998) and E2F-1 protein levels remain high during myoblast differentiation, despite a dramatic reduction in its mRNA (Martelli and Livingston, 1999).

14. Regulation of transcription by different E2F family members

The presence of at least 12 E2F·DP combinations in mammalian cells, together with the differences in their regulation and effects on cell growth noted above, would suggest that different E2F heterodimers have distinct transcriptional activities. However, identification of individual roles for different E2Fs is inherently difficult since they lie within an intricate regulatory network, and overexpression of individual members would be expected to have complex effects. For example, since the promoters of some E2F family members are themselves E2F-

responsive, overexpression of any E2F might be expected to lead to increased expression of other family members from the endogenous gene. Such effects of one E2F family member on another have been noted. Overexpression of both E2F-1 and E2F-4 leads to upregulation of E2F-1 expression (Pierce et al., 1998) and overexpression of E2F-1 activates the E2F-2 promoter (Sears et al., 1997) in REF52 cells. Similarly, in p16-arrested U343 astrocytoma cells, overexpression of E2F-1 induced expression of E2F-4 and overexpression of E2F-2 or E2F-4 led to increased expression of E2F-1 (Dirks et al., 1998). In addition to direct effects on E2F expression, overexpression may also affect E2F activity through effects on pocket proteins. Indeed, overexpression of E2F-1 has been shown to lead to pRB hyperphosphorylation (Shan et al., 1996b). Overexpressed E2Fs would also be likely to sequester pocket proteins; therefore, overexpression of a particular E2F would be expected to upregulate the activity of other E2Fs that bind the same pocket proteins. This may be of particular concern since overexpression of E2F-1 alters its pattern of phosphorylation and the phosphorylated forms specific to overexpression appear to preferentially bind to pRB (Yang and Sladek, 1997). Furthermore, since free E2F-4 and -5 are generally localized to the cytoplasm, effects of their overexpression may reflect sequestration of factors rather than a direct effect on transcriptional activity.

Despite these concerns, differences in the effects of overexpressing individual E2F family members have been seen at the level of gene expression. In studies of serum-starved REF52 cells, while overexpression of E2F-5 had little effect, overexpression of E2F-1, -2, -3 or -4 induced mRNA expression of a large variety of E2F-responsive genes (DeGregori et al., 1997). While some genes such as DNA polymerase α and cyclin E were induced essentially equally by all four active E2Fs, others were selectively activated. For example, cyclin A was only significantly induced by E2F-1 and -2 whereas DHFR and cdk2 were preferentially induced by E2F-2 and -3, respectively. In contrast, E2F-1, -2 and -3 are equally potent at activating orc1, cdc6 and Mcm mRNA in REF52 cells (Leone et al., 1998). In p16-arrested U343 cells, differences in the ability of E2F family members to induce different E2Fs and pocket proteins were seen. E2F-2 and -4 were each able to induce E2F-1, whereas E2F-1, but not E2F-2, could induce E2F-4. With respect to the pocket proteins, E2F-1 and -4 induced pRB, but only E2F-4 induced p130 and p107. Interestingly, in these cells, E2F-2 and -3 overexpression repressed expression of p130 but not p107 (Dirks et al., 1998). Consistent with promoter-specific effects of individual E2Fs, pocket proteins show a differential ability to regulate promoters (Hurford et al., 1997).

As with the apparent cell type-specific effects of E2F overexpression on growth and apoptosis, the selective nature of overexpression on gene expression also appears to be cell type/context-specific: in contrast to their relatively equivalent effects in serum-starved REF52 cells (DeGregori et al., 1997), E2F-2 induced cdk2 more potently than did E2F-3 in p16-arrested U343 cells (Dirks et al., 1998).

Functional analysis of E2F proteins has led to several observations that may explain these differential effects. As discussed above, the distribution of E2F family members between the nucleus and cytoplasm varies, which can explain some of their differential effects. However, subcellular localization cannot explain all of the observed differences. Although co-expression of DP1 with E2F-5 enhanced the ability of E2F-5 to induce S-phase in quiescent and p16-arrested Rat1 cells (Lukas et al., 1996), it did not enhance S-phase entry in REF52 cells, despite leading to an increase in nuclear localization of E2F-5 (DeGregori et al., 1997). Furthermore, E2F-1, -2 and -3 are all localized in the nucleus but show various abilities to induce different genes.

Some of the specificity in the promoter activation profiles of individual E2F family members may reside in their preferential binding to different promoter sequences. Although the residues of E2F that contact DNA in the core E2F site are conserved among the E2F family members (Zheng et al., 1999), such differences are becoming apparent. This is seen with the cyclin A promoter, where very weak binding by E2F-4 compared with E2F-1 has been implicated in its regulation (Liu et al., 1998) and correlates with its preferential activation by E2F-1 (DeGregori et al., 1997). Differential binding by individual E2Fs has also been detected using a PCR-based CASTing technique (Tao et al., 1997). This study revealed that, although the different E2F heterodimers recognize similar DNA sequences, they show different preferences for precise sequences and both the E2F and DP partner contribute to these sequence preferences. Although these studies give an interesting insight into potential mechanisms underlying the diverse effects of different E2Fs, further work is needed to determine how this relates to the activation of promoters by individual E2Fs, especially since half-life studies revealed that E2F-1 complexes bound more stably to all tested sequences than did any E2F-4 complexes. Nonetheless, these studies did demonstrate that altering the *B-myb* promoter to contain preferred sequences of different E2Fs altered its growth regulation. In light of the differences in the repressive and activating nature of E2F sites in different promoters, the finding that pRB changed the profile of preferred binding sites for E2F-1 is noteworthy since it indicates that pocket proteins may alter the promoter preference of E2Fs (Tao et al., 1997). Different E2F family members also show binding preferences for different promoters as revealed by formaldehyde crosslinking to the *DHFR*, *B-myb*, *cyclin E*, *cdc2*, *thymidine kinase* and *RB* promoters (Wells and Farnham, personal communication). These studies indicate that the particular E2F bound to a promoter varies

during the G_0 to S-phase transition in a promoter-specific manner that is not directly related to expression or localization. Therefore, factors other than the specific sequence of the E2F sites are also likely to underlie the promoter preference of individual E2Fs.

The less efficient ability of E2F-4 and -5 to enhance transcription may, in part, also lie in a weaker transactivation domain of these proteins; GAL4-E2F transactivation domain fusion proteins revealed that the transactivation domains of E2F-4 and -5 were less able to activate a GAL4-responsive promoter than that of E2F-1 (Pierce et al., 1998).

Another mechanism that may underlie the differing abilities of E2Fs to activate different promoters is their interaction with other factors. As noted above, E2F interacts with and cooperatively activates transcription with a number of different transcription factors and, as exemplified by Sp1, this can vary with different family members.

Analysis of DNA bending revealed that E2F sites have different intrinsic bend angles and E2F family members display differences in DNA bending activity (Tao et al., 1997). Therefore, since DNA bending has been implicated in interaction between promoter bound factors and in transcriptional activation by E2F (Huber et al., 1994; Cress and Nevins, 1996), it may also play a role in differential activation by different family members.

15. Regulation of transcription by E2F-6

E2F-6, a newly identified member of the E2F family, is significantly different from the other E2F family members in that it lacks transactivation/pocket protein-binding and cyclin A-binding domains (Morkel et al., 1997; Cartwright et al., 1998; Gaubatz et al., 1998; Trimarchi et al., 1998). In other regions, E2F-6 shares significant homology with other E2Fs and is able to form heterodimers with DP proteins which can bind to E2F sites. Despite this homology, E2F-6 does not simply act like an E2F protein with the cyclin A and transactivation domain deleted, since it does not cause the S-phase arrest in cycling NIH 3T3 cells seen with an E2F-1 mutant lacking these domains (Krek et al., 1995; Gaubatz et al., 1998). E2F-6 also differs from E2F-1 in its preference for the sequence of DNA in its binding site (Morkel et al., 1997; Cartwright et al., 1998). Furthermore, E2F-6 appears to contain a transcriptionally repressive domain because use of GAL4·E2F-6 fusion proteins revealed that human and mouse E2F-6 can actively repress transcription in SAOS-2 and HeLa cells, respectively (Morkel et al., 1997; Gaubatz et al., 1998). However, the precise nature of this repression remains to be elucidated since (a) E2F-6 did not show repressive activity in U2OS cells, and (b) the repressive domain has been mapped to different regions of mouse (N-terminus) and human (C-terminus) E2F-6 (Morkel et al., 1997; Cartwright et al., 1998; Gaubatz et al., 1998). Little information is available as to the physiological role of E2F-6; however, it can inhibit activation by E2F-1 through a mechanism that involves promoter competition (Morkel et al., 1997; Cartwright et al., 1998; Gaubatz et al., 1998; Trimarchi et al., 1998), it can increase the percentage of U2OS cells (but not SAOS2 or NIH 3T3 cells) in S-phase, and it inhibits S-phase entry when overexpressed in quiescent/serum-stimulated NIH 3T3 cells (Cartwright et al., 1998; Gaubatz et al., 1998).

In addition to its lack of interaction with cyclin A and pocket proteins, E2F-6 is unlikely to participate in many of the protein·protein interactions noted above since these are mediated by the E2F transactivation domain. It is, therefore, likely to be under a different set of control mechanisms from other E2F family members and clearly represents a novel tier in regulation of transcription by E2F. Information concerning the cell cycle expression of E2F-6 and interaction with the cell cycle machinery will help elucidate its role in modulation of E2F-dependent transcription.

16. Mechanisms underlying non-G_1/S regulation by E2F sites

Although the majority of E2F-regulated promoters are induced in late G_1, some E2F site-containing promoters can display different regulation. Studies with mouse E2F-1 and DHFR promoters have revealed that the ability of E2F to confer growth regulation is dependent on the position of the E2F sites and on the other factors binding to the promoter (Van Ginkel et al., 1996; Fry et al., 1997). Thus, promoter architecture and factor binding appear to be key aspects in regulation of transcription by E2F. Recently, two studies have indicated that such factors can also dictate the timing of the induction of E2F site-containing promoters. Although the *c-myc* promoter has been shown to be E2F-responsive and E2F sites are important for its expression in cycling cells, this promoter is induced with immediate–early kinetics. Its early induction has been attributed to a non-canonical binding site for STAT factors (signal transducers and activators of transcription) which overlaps the E2F site in the *c-myc* promoter (Kiuchi et al., 1999). In quiescent cells, the promoter is repressed by E2F·pocket protein complexes bound to the E2F sites; however, following stimulation of the cells through the gp130 receptor, STAT3 is activated and displaces E2F from the promoter. This leads to relief of pocket protein-mediated repression and activation of the promoter by STAT.

In contrast to *c-myc*, *cyclin A* is not induced until S-phase. The *cyclin A* promoter contains a CHR element that binds to the transcriptional repressor, CDF-1. The

timing of induction of *cyclin A* expression has been attributed to the interplay of the CHR element and an overlapping E2F site since they bind their respective factors in a mutually exclusive manner (Liu et al., 1998). Although E2F-4·p130 complexes have been shown to contribute to repression of *cyclin A* transcription in G_0 (Stiegler et al., 1998a), the E2F site shows marked preference for 'free' E2F-1 over 'free' E2F-4 and only E2F-1 is able to compete CDF-1 from the promoter (Liu et al., 1998). Therefore, prior to upregulation of E2F-1 in late G_1, CDF-1 binds to the promoter and represses transcription. As E2F-1 levels accumulate at G_1/S-phase, CDF-1 is displaced and *cyclin A* transcription is activated in S-phase.

Taken together these data indicate that both direct interactions of E2F with other factors and interactions through promoter architecture can have profound effects on regulation of transcription by E2F. Therefore, analysis of potential roles of E2F at a particular promoter will have to take into account specific features of promoter architecture as well as the direct regulation of E2F through protein·protein interactions.

17. Conclusions

In keeping with the central role of E2F in growth control, E2F activity is regulated by a network of overlapping mechanisms. In addition to regulation by pocket proteins, there are multiple pathways by which the cell can modulate E2F activity to obtain subtle changes in cell growth. However, while the basic consequences of the pocket protein regulation of E2F are fairly well characterized, the role of these other regulatory mechanisms in cellular growth control is less clear. In part, this confusion arises from studies in different cell types which have led to apparently opposite conclusions. Since growth of cells in culture requires aberrations in growth regulatory pathways which will differ for different cell lines, the ostensibly contradictory results are likely to reflect use of cells with different regulatory backgrounds. Therefore, full understanding of the network of pathways which converge on E2F to regulate cell growth will require careful analysis of the defects in individual cell lines. Indeed, comparison of how these defects affect E2F activity is likely to be a valuable tool in elucidation of the subtleties of these growth regulatory pathways.

Despite the continuing confusion, based on current knowledge of the regulation of E2F, a picture of how some of these additional regulatory mechanisms may superimpose on pocket protein regulation of E2F can be formulated. One tentative scenario for cycling cells in which E2F transactivation is limited to a very narrow window in G_1/S is as follows. In early G_1, E2F activity is likely to be repressed by mainly E2F-4·pRB complexes. As cells progress through G_1, these complexes are disrupted, E2F-4 relocalizes from the nucleus to the cytoplasm, and E2F-1, -2 and -3 levels increase. The net effect of these changes is increased 'free' E2F in late G_1 since phosphorylation of both E2Fs and pocket proteins inhibits their association. However, while the DNA binding of some of these E2Fs increases, that of E2F-1 is inhibited by phosphorylation of E2F-1 or DP1.

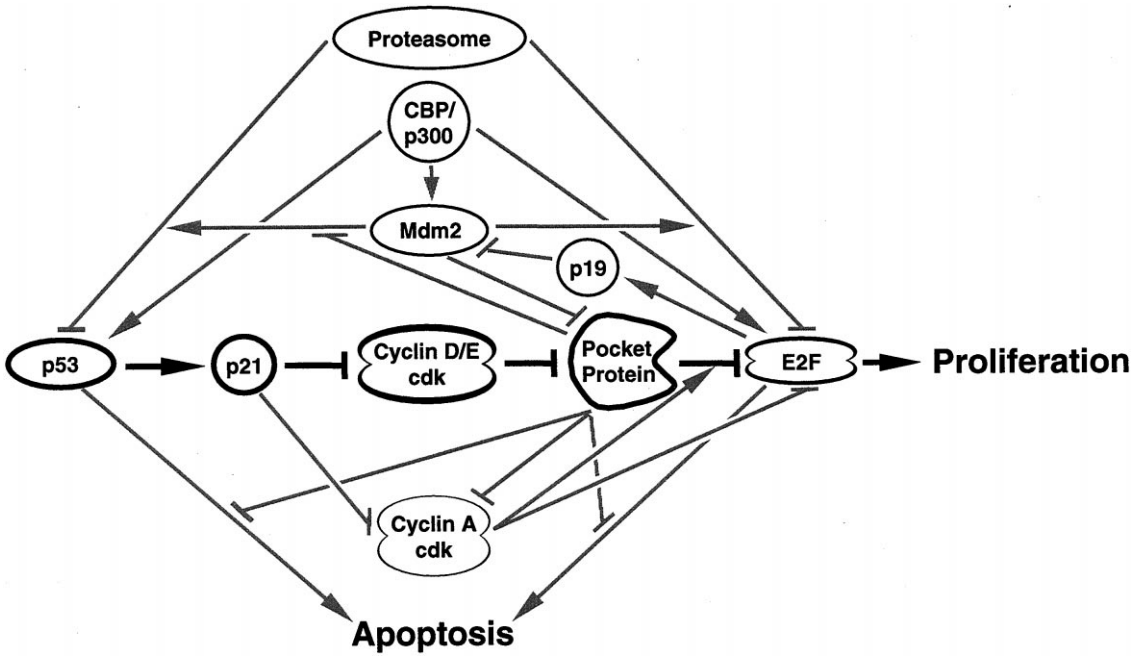

Fig. 2. Additional levels of regulation that could impinge on the control of E2F by p53. Arrowheads and bars indicate stimulatory and inhibitory effects, respectively. See text for references.

In S-phase, E2F activity is downregulated by phosphorylation-induced reduction in their DNA binding and by their degradation. In G_2/M, E2F-4 again relocates to the nucleus and represses transcription through association with pRB. At this time, degradation of E2F-1, -2 and -3 may be enhanced by the phosphorylation that inhibits their interaction with pocket proteins. When cells are induced to exit the cell cycle, p130 is induced and E2F-dependent transcription is repressed by E2F-4/5·p130 complexes. Although this scenario is consistent with known regulation of E2F, it remains an oversimplification. For example, potential roles of E2F-6 and many of the E2F interacting proteins have been ignored. Our current knowledge of pathways involving E2F makes it impossible to incorporate all of them into a single model in a review such as this. The extent of this complexity is diagrammed in Fig. 2, which illustrates how some of the mechanisms of E2F regulation described herein could impinge on the pathway by which p53 inhibits E2F activity through induction of $p21^{WAF1/CIP1}$ and inhibition of pRB phosphorylation.

From this review it is apparent that, although much has been learned about the role of E2F in the regulation of cell growth and cell cycle progression over the past decade, even more remains to be determined. Studies of E2F should yield exciting and valuable findings for many years to come.

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