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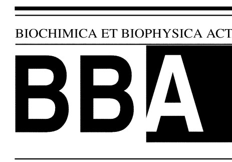

Mini-Review

E2F: a nodal point in cell cycle regulation

René Bernards *

Division of Molecular Carcinogenesis, Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, Netherlands

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1. Introduction

The transcription factor E2F was first identified as a factor that interacts with the adenovirus early region 2 (E2) promoter. Activation of the E2 promoter by the adenovirus E1A gene products was shown to be mediated through the E2F element and did not require novel protein synthesis [1]. Since these original observations, published approximately a decade ago, E2F has attracted considerable attention. Interest in E2F increased dramatically when it was found that E2F interacts with the retinoblastoma protein (pRb) and that this complex is disrupted by adenovirus E1A [2–5]. This observation provided the first insight into the mechanism by which pRb acts to inhibit cell proliferation. At the last count, the Medline database contained over 500 entries under ''E2F''. From these studies it has become evident that E2F, apart from its role in regulation of adenovirus early gene expression, is a nodal point in mammalian cell cycle regulation. This review will summarize the way in which E2F transcription factors contribute to cell cycle regulation, how E2F activity is controlled during progression through the cell division cycle and how E2F activity is deregulated in cancer.

* Corresponding author. Fax: 31 20 512 1954; E-mail: bernards@nki.nl

2. Components of the E2F regulatory network

The finding that E2F interacts with the retinoblastoma protein provided a strategy to isolate molecular clones for E2F. Using pRb as a probe to screen cDNA expression libraries, a cDNA was isolated encoding a protein with E2F-like properties, named E2F-1 [6–8]. Purification of E2F DNA binding activity from HeLa cells revealed that high affinity DNA binding requires two components, suggesting that E2F DNA binding activity consists of a heterodimer [9]. Indeed, a second component was purified and cloned from E2F complexes, named DP-1, which turned out to cooperate with E2F-1 in DNA binding and trans-activation of E2F-site-containing promoters [10–12].

It is now evident that E2F-1 and DP-1 are founding members of gene families (Fig. 1). Till date, five E2F-like polypeptides have been identified and in spite of confusing nomenclature probably only 2 genes encode DP-like proteins [13–22]. E2F DNA binding activity is not only found in complex with pRb but also with two pRb homologues, p107 and p130, collectively known as the pocket proteins. The E2F component rather than the DP component of the heterodimer specifies the preference for pocket protein binding: E2F-4 interacts preferentially with p107 and p130, but under certain conditions also with pRb. E2F-5 binds p130 only and E2F-1, -2 and -3 interact

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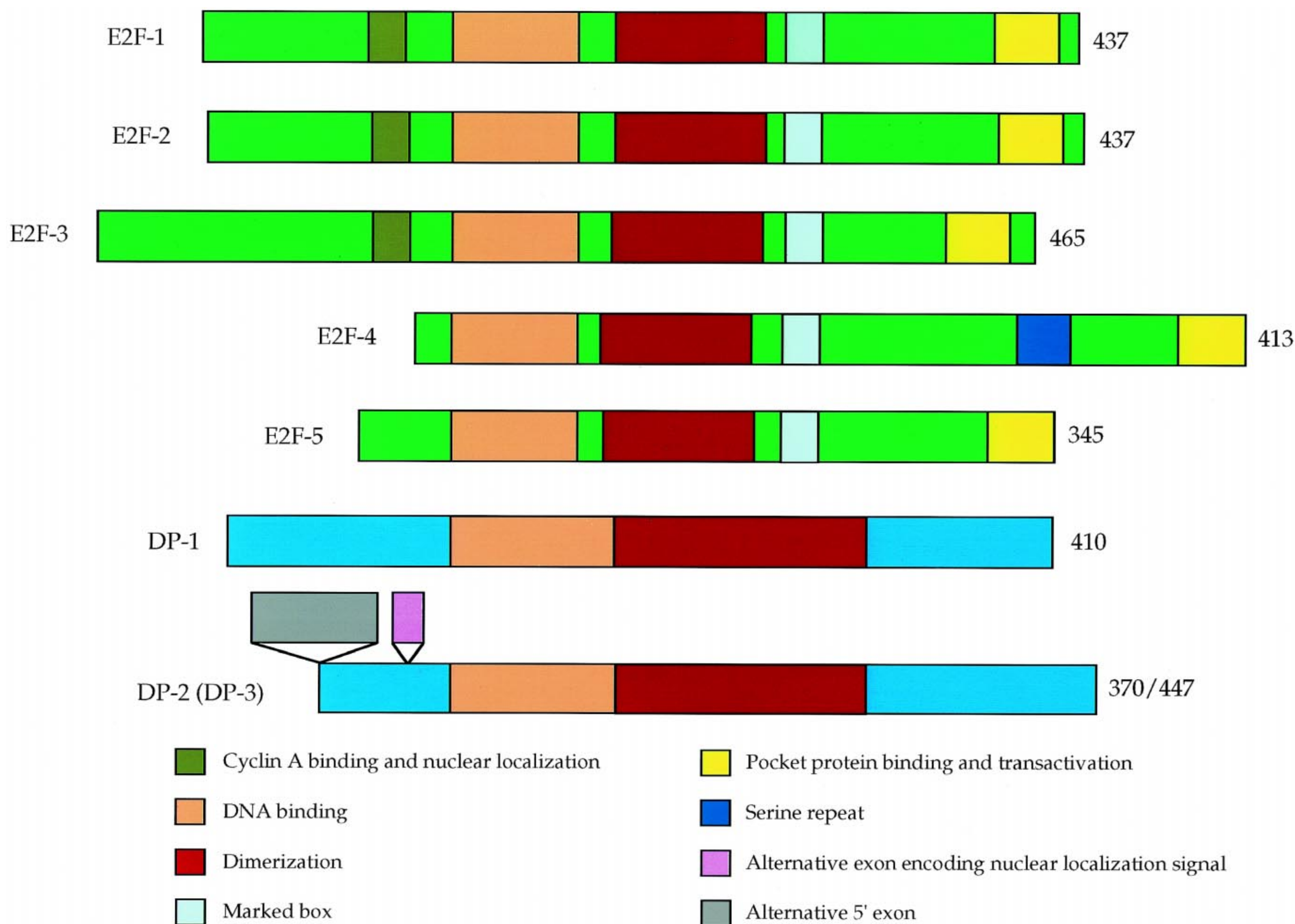

Fig. 1. Schematic representation of the E2F/DP family of transcription factors. The functionally defined and conserved motifs of the proteins are indicated.

exclusively with pRb [15–17,19,23,24]. The various E2F/pocket protein complexes are thought to perform different tasks as they are present at different stages of the cell cycle. In spite of this difference in appearance it is likely that there is also a degree of functional redundancy between the various E2F/pocket protein complexes. For instance, mice that lack either p107 or p130 develop normally, whereas p107; p130 double knock out mice die around birth from severe defects in bone formation [25,26]. Similarly, embryos homozygous for disruption of both *Rb* and p107 die at day 11.5 of gestation, two days earlier than embryos that lack *Rb* only [25]. Both examples illustrate that, during development, pocket proteins can partially compensate for each other. Since E2Fs are thought to be the most relevant downstream targets of p107 and p130, these data suggest that the E2F/pocket protein complexes also show functional redundancy during development.

Very recently, an E2F-like protein, named EMA, was isolated based on its ability to interact with DP-1. EMA differs from E2F however in that it lacks a transactivation domain and pocket protein binding surface. Instead, it appears to contain a potent N-terminal repression domain (T. Kouzarides and C. Hagemeier, personal communication). It is not clear at present whether EMA contributes to regulation of E2F target genes or not.

3. Cell cycle regulation of E2F

An important level of E2F regulation is imposed by the pocket proteins. Binding of hypophosphorylated pocket proteins to their respective E2F partners causes inhibition of transactivation. Phosphorylation of pocket proteins by cyclin/cyclin-dependent kinase (CDK) complexes dissociates the inhibitory pocket protein, releasing E2F as an active transcription factor. Not all pocket proteins are inactivated through phosphorylation by the same cyclin/CDK complexes. For instance, p107 is phosphorylated and inactivated only by cyclin D1/CDK4, and not by cyclin E/CDK2 or cyclin A/CDK2, whereas all three kinase complexes can phosphorylate and inactivate pRb [27,28]. In addition, expression levels of two of the pocket proteins are differentially regulated during the cell cycle. Whereas in most cell types pRb protein levels are invariant, p107 levels increase at the G1- to S phase transition, and p130 levels decline in S phase [27,29]. This, together with the fact that mRNA expression of most E2Fs increases in late G1 [18], causes E2F complexes to undergo dynamic changes when cells progress through a cell cycle. Complexes consisting of E2F and p130 are found predominantly in quiescent cells and in terminally differentiated cells, E2F/p107 complexes are present mostly in S phase and E2F/pRb in early to mid G1. A considerable increase in free E2F is seen in late G1, coincident with the initial phosphorylation of the pocket proteins, leading to the coordinated activation of a group of S phase specific genes (see below and Ref. [30] for a more extensive review).

The cyclin/CDK complexes not only regulate E2F through phosphorylation of the pocket proteins. Phosphorylation of the DP component of the E2F complex by cyclin A/CDK2 has been shown to cause down-regulation of E2F DNA binding activity in S phase [31,32]. This phosphorylation is thought to play a role in down-modulation of S phase-specific genes when DNA replication is well under way.

A new level of regulation of E2F activity was recently described by several groups [33–36]. E2F-1 -2 and -3 localize constitutively to the nucleus, whereas E2F-4 and -5 are cytosolic. Nuclear translocation of E2F-4 or -5 requires co-expression of their pocket protein partner. Apparently, E2F-4 and -5 lack a nuclear localization signal and can only piggy back into the nucleus when in complex with a protein that does [33–36]. Consistent with this, E2F-4 is mostly nuclear in quiescent cells where E2F-4 is in pocket protein complexes and shifts to the cytosol in S phase, when the pocket proteins become phosphorylated [34]. Poor nuclear transport is also seen when E2F-4 or -5 are expressed with DP-1, which lacks a nuclear localization signal. In contrast co-expression of DP-2 (also known as DP-3) does cause efficient nuclear transport of E2F-4. Interestingly, the domain of DP-2 that harbors the nuclear localization signal is subject to alternative splicing, indicating that multiple DP-2 isoforms exist that differ in their ability to co-transport E2F proteins to the nucleus [33] (see also Fig. 1). At present, no information is available to indicate that DP-2 RNA splicing is regulated during the cell cycle or during differentiation.

In addition to the levels of regulation described above, it is now clear that targeted protein degradation is also used to control E2F protein levels [37–39]. Free E2F transcription factors are unstable and rapidly degraded by the ubiquitin-proteasome pathway through a carboxyl-terminal destabilization signal. E2Fs in pocket protein complexes are protected from degradation, possibly through shielding of the destabilization signal by the pocket protein. It is likely that this level of regulation exists to limit E2F activity after release from inhibitory complexes.

4. Cell cycle regulation by E2F

The five E2Fs differ significantly in their ability to induce S phase in quiescent cells. The pRb-interacting E2F-1, -2 and -3 by themselves strongly induce cell cycle entry, whereas E2F-4 only has weak S phase-promoting activity and requires co-expression of DP-1 for growth-promoting activity [15,40–44]. The weak growth-stimulatory activity of E2F-4 is most likely the result of the absence of a nuclear translocation signal as tagging of E2F-4 with a nuclear translocation signal greatly increases its ability to induce S phase entry [36]. Nevertheless, E2F-1, -2, -3 and -4 can transform cells in culture [15,42,45–47]. E2F-5 likely does not possess transforming activity and in agreement with this, is also the only E2F that lacks the ability to induce S phase in quiescent cells [48]. However, E2F-5 expression is induced strongly

when cells progress from G1 to S in response to serum stimulation, suggesting a positive role for E2F-5 in cell cycle progression [18]. Over-expression of DP-1 and DP-2 also causes cells to progress from G1 to S [15,49] and both have transforming activity when co-transfected with an activated *ras* oncogene. Analysis of transforming activity of DP-1 mutants indicated that the primary target through which DP-1 mediates its oncogenic activity is unlikely to be due to the regulation of E2F site-transcription, suggesting an E2F-independent effector function for DP-1 [50].

Besides stimulation of S phase entry, E2F-1 can induce apoptosis [41–43]. Recent evidence indicates that induction of S phase can be uncoupled from apoptosis induction: E2F-1 and -2 are equally effective in induction of S phase, but of all five E2Fs tested, only E2F-1 was found to induce apoptosis [48].

That E2F is a critical regulator of cell cycle progression, was also demonstrated using dominant-negative mutants of DP-1. Expression of mutants of DP-1 that retain E2F dimerization activity but lack DNA-binding activity cause cycling cells to arrest in G1 [49]. Furthermore, ectopic expression of E2F-1 blocks terminal differentiation and causes proliferation in transgenic megakaryocytes [51]. Finally, mice that lack E2F-1 display atrophy of certain cell types, most likely as a result of insufficient stimulation of cell proliferation [52,53]. Together, these data provide strong evidence that E2F can stimulate progression through the cell cycle. The activities of the various E2F components are summarized in Table 1.

5. E2F-regulated genes

In broad terms, E2F target genes fall into two categories: those whose encoded proteins are required for synthesis and replication of DNA and those that contribute to cell cycle regulation. The first category includes dihydrofolate reductase, thymidine kinase, DNA polymerase α and a component of the Origin Recognition Complex (ORC). The second class includes cyclin A, cyclin E, p107, *Rb* and E2F-1 and the nuclear proto-oncogenes c-*myc*, N-*myc* and B-*myb* [30]. The observed accumulation of free E2F in late G1 was taken to mean that its major role is to activate a set of genes during that period. An unexpected finding therefore was that deletion of the E2F element in the B-*myb* promoter did not interfere with induction of B-*myb* in S phase but rather caused de-repression in G1 [54]. This demonstrated that in the B-*myb* promoter the E2F element acts as a negative element in G1 rather than as a positive element in S phase. Indeed, subsequent analysis of other promoters revealed that E2F elements act in most cases as negative elements and only rarely as positive elements. These differences in transcriptional effects depend most likely on the promoter context.

It is likely that the five known E2Fs control the expression of distinct sets of target genes. For example, fibroblasts that lack *Rb* manifest different changes in expression of E2F-responsive genes than cells that lack both p107 and p130 [55]. Among the best-characterized promoters in which E2F acts as a positive element is the promoter for cyclin E [56–59].

Table 1
Properties of E2F and DP transcription factors

E2F component	Pocket protein partner	Active during	Complexed in	S phase induction	Nuclear localization	Apoptosis induction
E2F-1	pRb	late G1–mid S	Early-mid G1 (pRb)	+ + +	Yes	+ + +
E2F-2	pRb	late G1–mid S	Early-mid G1 (pRb)	+ + +	Yes	−
E2F-3	pRb	late G1–mid S	Early-mid G1 (pRb)	+ + +	Yes	−
E2F-4	p107, p130 (pRb) [a]	late G1–mid S	Go (p130); S (p107)	+	No	−
E2F-5	p130	late G1–S (?)	Go (p130)	−	No	−
DP-1	pRb, p107, p130	G1–mid S	Constitutive	+	No	−
DP-2/DP-3 [b]	pRb, p107, p130	?	Constitutive	+	Yes [c]	−

[a] Lower affinity for pRb compared to p107 and p130.
[b] DP-2 and DP-3 are most likely two names for the same gene product.
[c] Only certain splice variants.

It is likely that E2F activates this promoter to create an auto-stimulatory loop: initial release of active E2F-1 from pocket protein complexes by cyclin D1/CDK4 causes an increase in cyclin E transcription. Upon assembly of cyclin E/CDK2, additional phosphorylation of pRb occurs, which in turn leads to a further increase in free E2F-1, which jump-starts a program of late G1- and S phase-specific transcription.

E2F elements can act as negative elements through recruitment of pocket proteins, which have dominant repression activity [60,61]. This is illustrated by the de-repression of B-*myb* in quiescent fibroblasts with targeted disruption of both p107 and p130, which most likely results from loss of E2F/pocket protein complex-mediated repression [55]. The precise mechanism through which pocket proteins repress transcription is not clear. However, the pocket appears to be involved in recruitment of transcription-inhibitory proteins as phosphorylation or mutation of the pocket abolishes repression [62–65]. Recent data also implicate cyclin E/CDK2 complexes (which can bind to p107 in E2F complexes) in regulation of certain E2F element-containing promoters. For example, the cyclin A promoter is activated strongly by cyclin E/CDK2 and this activation is mediated through the E2F element. Since cyclin E/CDK2 does not disrupt the E2F/p107 complex bound to this promoter, it is possible that E2F/p107 complexes recruit cyclin E/CDK2 to the promoter where it acts to phosphorylate other factors that bind in close proximity to the E2F site [66].

Since E2F-pocket protein complexes (mostly E2F-4/p130) are abundant in quiescent cells it has been suggested that they contribute to maintenance of quiescence by preventing expression of cell cycle-associated genes in Go. Indeed, the finding that free E2F-4 is mostly cytosolic and pocket protein-bound E2F-4 is nuclear would seem consistent with a primary role for E2F-4/pocket protein complexes in transcriptional repression [33,34]. On the other hand, over-expression of E2F-4 causes cell cycle progression and transformation, which suggests that free E2F-4 also has a positive role in cell cycle regulation [15,16]. The unexpected phenotype of mice lacking E2F-1 could also be taken as evidence that E2F/pocket protein complexes prevent cell cycle entry. Whereas some tissues (notably testes) show atrophy, several others (e.g. thymus) are hyperplastic and at later age even become neoplastic [52,53]. The hyperplasia might be explained by a loss of repression of growth-promoting genes. However, if this model is correct, it is surprising that mice that lack both p107 and p130 (which should result in a more significant reduction in E2F-pocket protein complexes in Go) have so few defects in cell proliferation [26]. A more likely scenario may therefore be that loss of E2F-1 causes hyperplasia by preventing execution of an E2F-1-dependent apoptosis program in certain tissues [53].

6. Conclusions

The upstream regulators of E2F, pRb, cyclin D1 and $p16^{INK4A}$ (a specific inhibitor of CDK4 and CDK6) are frequently affected in human cancers. Mutations in *Rb* occur not only at near 100% frequency in retinoblastoma but also in a variety of other malignancies. Overexpression of cyclin D1 is found in more than 50% of breast cancers, the relevance of which is underscored by the finding that over-expression of cyclin D1 in the breast epithelium of transgenic mice predisposes to breast cancer [67]. Finally, loss of $p16^{INK4A}$ not only predisposes to melanoma but is also frequently found in several sporadic cancers [68]. Only few forms of cancer consistently lack mutations in the $p16^{INK4A}$-cyclin D1-pRb pathway. However, the recent demonstration that the retinoblastoma protein is a critical downstream target of Ras-dependent signaling pathways indicates that the $p16^{INK4A}$-cyclin D1-pRb pathway can also be functionally inactivated without being mutated itself [69]. Because pRb, cyclin D1 and $p16^{INK4A}$ are all upstream regulators of E2F activity, the frequent involvement of these proteins in human cancer suggests a central role for E2F in control of cell proliferation. Surprisingly, alterations in E2F itself in human cancer are rare indeed. Only sporadic cases of amplification have been observed [70]. It is possible, however, that point mutations in E2F which abolish pocket protein interaction [71] are present in some forms of cancer.

It is important to bear in mind that the $p16^{INK4A}$-cyclin D1-pRb pathway is not a linear pathway that signals to E2F only. For instance, $p16^{INK4A}$ does not

only act to inactivate cyclin D1/CDK4, but its deletion also causes activation of cyclin D2 and D3-associated kinases, which may act on other substrates. Furthermore, cyclin D1 expression does not only cause inactivation through phosphorylation of pRb, but also of p107 and p130. That p130 does contribute to growth control was recently shown. In one of seventeen small cell lung cancer cell lines, a splice acceptor site in the gene encoding p130 was mutated [72]. Consistent with a role for p107 and p130 in growth control is also the finding that the transforming proteins of several DNA tumor viruses bind and inactivate all three pocket proteins [1]. In addition, Zwijsen et al. have recently found that cyclin D1 has a role in breast cancer that is completely independent from its role as a CDK activator. Through direct binding to the estrogen receptor, cyclin D1 can mediate ligand-independent activation of the estrogen receptor [73]. Finally, pRb has other targets in addition to E2F, such as PU-1, Elf-1, ATF-2, MDM2 and MyoD [30]. Together, these data suggest strongly that $p16^{INK4A}$ loss, cyclin D1 over-expression and inactivation of pRb are not equivalent. Consistent with this, mutation of these genes is found in distinct forms of human cancer. Importantly, loss of $p16^{INK4A}$ and pRb is sometimes found in the same tumor, indicating that loss of additional genes in the pathway confers an additional growth advantage [74]. Together, these data indicate that $p16^{INK4A}$, cyclin D1 and pRb have targets in addition to E2F that mediate (part of) their effects on cell proliferation. Nevertheless it is likely that, in tumors with a defect in the $p16^{INK4A}$-cyclin D1-pRb pathway, enhanced activity of E2F does contribute to the malignant phenotype. However, the surprising finding that loss of E2F-1 in mice results in a tumor-prone phenotype, forces us to reconsider the simplistic model that E2F only acts to promote cell division. That E2F-1 can act both as an oncogene and a tumor suppressor gene teaches us an important lesson: if we think that we understand E2F after a decade of E2F research, we are likely to be proven wrong in the next decade.

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