E2F-4, a new member of the E2F gene family, has oncogenic activity and associates with p107 in vivo

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The E2F family of transcription factors controls the expression of genes that are involved in cell cycle regulation. E2F DNA-binding activity is found in complex with the retinoblastoma protein, pRb, and with the pRb-related p107 and p130. To date, cDNAs for three members of the E2F gene family have been isolated. However, all three E2Fs associate in vivo exclusively with pRb. We report here the cloning and functional analysis of a fourth E2F family member. E2F-4 encodes a 413-amino-acid protein with significant homology to E2F-1. E2F-4 antibodies recognize a 60-kD protein in anti-p107 immunoprecipitates, indicating that E2F-4 associates with p107 in vivo. Like the other E2Fs, E2F-4 requires DP-1 for efficient DNA binding and transcriptional activation of E2F site-containing promoters. Increased expression of E2F-4 and DP-1 in SaoS-2 osteosarcoma cells causes a shift from G1-phase cells to S and G2/M-phase cells, suggesting a role for E2F-4 in regulation of cell-cycle progression. We show that expression of E2F-4 and DP-1 together with an activated ras oncogene in rat embryo fibroblasts, causes transformation, indicating that E2F-4 has oncogenic activity.

[Key Words: E2F; p107; oncogene; cell cycle]

Received August 5, 1994; revised version accepted September 23, 1994.

The E2F gene family encodes a number of closely related transcription factors that control gene expression during the cell division cycle. E2F sites have been found in a number of genes whose expression is tightly regulated during the cell cycle (for review, see Nevins 1992; Farnham et al. 1993). Importantly, in a number of cases, it has been shown that mutation of the E2F-binding sites in the promoters of these genes leads to a loss of cell-cycle-regulated expression (Farnham et al. 1993; Lam and Watson 1993). Complexes have been found between E2F and the retinoblastoma protein (pRb), E2F and the pRb-related p107, and E2F and a third member of the pRb gene family, p130. These three complexes show a different pattern of appearance during the cell cycle. Complexes between E2F and pRb are found mostly in the G1 phase of the cell cycle, but some E2F/pRb complexes persist during S phase. The complexes between E2F and p107 show a more complex pattern of appearance during the cell cycle. In G1, DNA-binding complexes have been observed that contain E2F, p107, cyclin E, and cyclin-dependent kinases (cdk), cdk2. In S phase cyclin E is no longer found in these complexes, but cyclin A is found associated with E2F, p107, and cdk2 (Lees et al. 1992; Shirodkar et al. 1992). The function of these cyclin/cdk complexes in the E2F/p107 complex remains obscure as yet. In addition, complexes have been observed between E2F and p130 primarily in quiescent cells (Cobrinik et al. 1993). E2F/p130 complexes disappear quickly when cells emerge from quiescence and enter into the cell cycle.

The activity of E2F is tightly regulated by association with proteins of the pRb gene family. Two findings were critical for our understanding of the mechanism by which pRb regulates E2F activity. First, the demonstration that pRb can inhibit trans-activation by E2F showed that proteins of the pRb gene family act as negative regulators of E2F activity (Chellappan et al. 1991; Hamel et al. 1992; Hiebert et al. 1992; Weintraub et al. 1992, Helin et al. 1993a). Second, the demonstration that E2F binds preferentially to hypophosphorylated pRb suggested that complex formation between pRb and E2F is regulated by phosphorylation of pRb (Chellappan et al. 1991; Lam and Watson 1992; Hiebert et al. 1992; Weintraub et al. 1992, Helin et al. 1993a). Because pRb can be phosphorylated by cyclin E and the D-type cyclins, when in complex with their respective cdk, these cyclin/cdk complexes are thought to release E2F from pRb by phosphorylating pRb. Recently, a second cyclin/cdk complex was shown to act as a negative regulator of E2F activity: Cyclin A can bind directly to E2F-1 and inhibit DNA binding by E2F-1 [Krek et al. 1994]. As cyclins D and E are present primarily in late G1 and cyclin A primarily in
S phase, E2F-1 activity is likely to increase in late G1 and decline in S phase when cyclin A is synthesized. It has been shown that p107, like pRb, can inhibit E2F-dependent gene expression (Schwarz et al. 1993; Zamanian and La Thangue 1993; Zhu et al. 1993). However, very little is known about the regulation of the E2F activity that interacts with p107.

E2F DNA-binding activity consists of a heterodimeric complex of two molecules: An E2F polypeptide and a dimerization partner named DP-1. cDNAs encoding E2F-1 and DP-1 have been isolated by molecular cloning (Helin et al. 1992; Kaclin et al. 1992; Girling et al. 1993; Huber et al. 1993). Recently, the complexity of this intricate network of interacting proteins increased further when two homologs of E2F-1, named E2F-2 and E2F-3, were isolated (Ivey-Hoyle et al. 1993; Lees et al. 1993). E2F-1, E2F-2, and E2F-3 associate in vivo only with pRb and not with the related p107. The presence of E2F activity associated with p107 (Cao et al. 1992; Lees et al. 1992, Shirodkar et al. 1992) would indicate that the family of E2F-like polypeptides is even larger and includes additional members that have the ability to associate with p107. Consistent with this hypothesis, it has been shown that p107-associated E2F polypeptides are similar, but distinct from pRb-associated E2F polypeptides (Dyson et al. 1993). We report here the cloning of a novel member of the E2F gene family, E2F-4, that interacts with p107 in vivo. We show that E2F-4 can promote cell-cycle progression and can act as an oncogene when overexpressed in primary fibroblasts.

**Results**

**Isolation of cDNA clones encoding E2F-4**

To isolate cDNAs encoding proteins that interact with p107, we screened a day-16 whole mouse embryo cDNA expression library with a 32P-labeled p107 protein probe. Three independent phage were isolated whose encoded fusion protein bound to p107. Partial DNA sequence analysis revealed that all three were derived from the same gene. The shortest clone had a cDNA insert of 800 bp and contained an open reading frame of only 31 amino acids. These 31 amino acids showed significant homology (15 of 31 identities) to the pRb-binding domain of E2F-1. The longer cDNA clones showed additional homology to the three known E2Fs. Because of this, we named the protein encoded by this novel cDNA, E2F-4. The partial mouse E2F-4 cDNA clones were then used to obtain longer mouse cDNAs and to isolate a human E2F-4 cDNA. Despite extensive efforts, we were unable to obtain the extreme 5' end of the human E2F-4 cDNA. We did, however, obtain a full-length mouse E2F-4 cDNA. Comparison of the 5' ends of the mouse and human E2F-4 cDNAs indicated that the human cDNA only lacked the first 16 codons of E2F-4. These 16 amino acids, however, are fully conserved between mouse and human E2F-4 (Ginsberg et al., this issue). Translation of the human E2F-4 cDNA (including the first 16 amino acids derived from mouse E2F-4) yields a 413-amino-acid protein with a predicted molecular mass of 44 kD.

Figure 1A shows the E2F-4 cDNA sequence and the deduced amino acid sequence. E2F-4 and E2F-1 share three regions of homology (Fig. 1B). The overall similarity between the two proteins is 63% (41% identity). A striking difference between E2F-4 and E2F-1 is that E2F-1 has an additional 100 amino acids amino-terminal of the DNA-binding domain. This region of E2F-1 contains a cyclin A-binding site (amino acid residues 67–108; Krek et al. 1994). The first region of homology is from amino acids 10–82 of E2F-4 and 120–190 of E2F-1 (similarity 71%), which coincides with the DNA-binding region of E2F-1 (Helin et al. 1993b). E2F-4 can only bind DNA in a heterodimeric complex with DP-1. The region required for DP-1 interaction is located downstream of the DNA-binding domain (amino acids 191–284). The similarity between E2F-4 and E2F-1 in this region is ~64%. E2F-4 and E2F-1 diverge in the carboxyl terminus. Downstream of amino acid 194 of E2F-4 and 297 of E2F-1 there is a region with almost no sequence conservation. In this region, E2F-4 contains a remarkable stretch of 12 consecutive serines. Although E2F-1 does not contain this stretch of serines, it does contain a region that is serine-rich. The functional significance of this repeat in E2F-4 is presently unknown. The last segment of homology is located at the extreme carboxyl terminus of the two proteins. This region of homology corresponds to the region of E2F-1 that is involved in binding to pRb (Helin et al. 1992). Because the first E2F-4 cDNA that we isolated with the p107 protein probe encoded only the last 31 amino acids of E2F-4, it is likely that this region, like its counterpart in E2F-1 that is sufficient for pRb binding, is sufficient for binding to p107. In this region, 15 of the 31 residues are identical between E2F-4 and E2F-1.

To analyze the pattern of expression of E2F-4, a partial mouse E2F-4 cDNA was used to probe a Northern blot containing poly(A)+ RNA from various tissues of the mouse. At high stringency, a single transcript of 2.1 kb was detected. The expression was high in kidney and thymus and relatively low in lung, brain, spleen, and testis (data not shown).

**E2F-4 requires DP-1 for DNA binding**

Efficient sequence-specific DNA binding by E2F-1 requires heterodimerization with a second protein named DP-1 (Helin et al. 1993b; Krek et al. 1993). To investigate whether E2F-4 can function like the other members of the E2F gene family, we asked whether E2F-4 was able to bind an E2F DNA consensus site, and whether DP-1 was required for DNA binding by E2F-4. We transfected U2-OS osteosarcoma cells with DP-1 and E2F-4 expression vectors separately or together. After 2 days, gel shift extracts were made from the transfected cells, and these extracts were incubated with a 32P-labeled oligonucleotide that specifies a consensus E2F site. DNA–protein complexes were then resolved on an acrylamide gel and detected by autoradiography.

As can be seen in Figure 2, transfection of U2-OS cells with DP-1 alone did not lead to an increase in E2F DNA-binding activity. Transfection of E2F-4 expression vector...
alone, however, yielded a clearly detectable increase in E2F activity. The most striking increase was seen when E2F-4 and DP-1 expression vectors were introduced simultaneously in the osteosarcoma cells (Fig. 2). These results show that E2F-4 can bind to a consensus E2F site and that binding is enhanced in the presence of DP-1. E2F-4 and DP-1 are synergistic in the activation of an E2F site reporter

Next we asked whether E2F-4 has the ability to activate the transcription of a CAT reporter gene that was linked to a core promoter and four upstream E2F sites. We cotransfected C33A cervical carcinoma cells with increasing amounts of E2F-4 (Fig. 3A) or DP-1 expression vectors (Fig. 3B) and the CAT reporter plasmid. When transfected alone, E2F-4 was unable to activate transcription of the CAT reporter. In contrast, expression of DP-1 led to a clearly detectable increase in the activity of the reporter gene. When E2F-4 and DP-1 were cotransfected, a strong increase in transcriptional activity was observed. The activation by E2F-4 and DP-1 together was more than the sum of either plasmid alone, indicating that E2F-4 and DP-1 are cooperative in trans-activation. The cotransfection of E2F-4 and DP-1 with a reporter plasmid containing a mutant E2F site did not result in increased activation of the reporter (data not shown). Together, these experiments demonstrate that E2F-4 is able to activate transcription from a promoter containing E2F DNA-binding sites in the presence of DP-1.

E2F-4 and DP-1 are synergistic in the activation of an E2F site reporter

Figure 1. [A] Nucleotide sequence of the E2F-4 cDNA. The first 74 bp was derived from mouse E2F-4, and the remainder from human E2F-4. [B] Schematic representation of E2F-4 in comparison with E2F-1. Black and shaded boxes are regions of high homology; hatched regions are specific for E2F-4 or E2F-1.
Expression of wild-type p107 or pRb and not by a mutant form of p107 protein that lacks growth-inhibitory activity (Zhu et al. 1993).

**E2F-4 specifically interacts with p107 in vivo**

In nontransfected cells E2F-1 interacts only with pRb and not with p107 (Dyson et al. 1993; Lees et al. 1993).

**Figure 2.** E2F-4 requires DP-1 for DNA binding. U2-OS cells were transfected with expression plasmids as indicated above each lane, and cell extracts were prepared for gel retardation assays as described in Material and methods. Cell extracts were tested for binding to a ³²P-labeled oligonucleotide that specifies a consensus E2F DNA-binding site.

**Figure 3.** E2F-4 and DP-1 cooperate in trans-activation. (A) C33A cells were transfected with increasing amounts of pCMV-E2F-4 (lower curve) or with increasing amounts pCMV-E2F-4 together with 100 ng of pCMVDP-1 (upper curve) together with 2 μg of reporter construct (E2F₄CAT) and 0.2 μg of RSV luciferase as an internal control. CAT activity was normalized to the luciferase activity for each sample [corrected counts]. CAT activities are the average of duplicate samples. (B) C33A cells were transfected as described in A, except that pCMVDP-1 was transfected in increasing amounts (lower curve) or increasing amounts of pCMVDP-1 in combination with 100 ng of pCMV-E2F-4 (upper curve). CAT activity was calculated as in A.

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**p107 inhibits E2F-4 trans-activation**

Transcriptional activation by E2F-1 can be suppressed by pRb, because the pRb-binding site of E2F-1 and the trans-activation domain overlap. Because E2F-4 was isolated with a p107 protein probe, we studied the effect of p107 on E2F-4 trans-activation. C33A cervical carcinoma cells were cotransfected with E2F-4 and DP-1 expression vectors and the E2F-site containing CAT reporter plasmid in the presence of increasing amounts of p107 expression vector. Figure 4 shows that p107 can efficiently suppress E2F-4 trans-activation. Maximal inhibition was already observed with 200 ng of p107 expression vector. As a control we used the p107 mutant, p107DE, that lacks most of the pocket region of p107 (Zhu et al. 1993). Cotransfection of 1 μg of pCMVp107DE did not significantly affect E2F-4 trans-activation (Fig. 4). When pRb was expressed, a decrease in E2F-4-mediated trans-activation was observed, although to a lesser extent than with p107. Inhibition of E2F-mediated trans-activation by transfection of either p107 or pRb has been observed previously (Zamanian and La Thangue 1993; Zhu et al. 1993), indicating that both pocket proteins are able to associate with endogenous E2F's when transiently overexpressed. These experiments, however, show that trans-activation by E2F-4 can be suppressed by overex-
Figure 4. p107 inhibits E2F-4-mediated trans-activation. C33A cells were transfected with 100 ng of pCMV-E2F-4 and 100 ng of pCMV-DP-1 in combination with increasing amounts of pCMV-p107, 100 ng of pRb, or 1000 ng of pCMV-p107-DE. Together with the expression plasmids, the cells were transfected with 2 μg of E2F,CAT and 0.2 μg of pRSV luciferase. CAT activity was normalized to the luciferase internal control. Fold activation was calculated relative to the basal level of E2F,CAT, which was set to unity (1.0). Data are representative of at least three independent experiments performed in duplicate.

However, when E2F-1 and p107 are transiently transfected, an interaction can be observed (R.L. Beijersbergen and R. Bernards, unpubl.). This indicates that overexpression of these proteins may lead to interactions that are not observed under physiological conditions. E2F-4 was identified by virtue of its ability to bind p107 in vitro. The data in Figure 4 suggest that E2F-4 can interact with p107 and pRb in transiently transfected cells, but they did not show that these interactions also take place under physiological conditions. To address whether E2F-4 interacts with p107 and/or pRb in nontransfected cells, we generated E2F-4-specific polyclonal antiserum and used this in a sequential immunoprecipitation experiment. ML-1 leukemia cells were metabolically labeled with [32P]orthophosphate. Nonionic detergent lysates were prepared and subjected to immunoprecipitation with either p107-specific monoclonal antibody or pRb-specific antibody. Proteins that were coimmunoprecipitated with pRb and 107 were then released by heating in SDS-containing buffer, diluted, and reimmunoprecipitated with polyclonal E2F-4 antiserum, E2F-1-specific monoclonal antibody, or nonimmune control serum. Consistent with the data of others (Dyson et al. 1993; Lees et al. 1993) mAb E2F-1 recognized a single protein species that is present in the anti-pRb immunoprecipitate but not in the anti-p107 immunoprecipitate (Fig. 5). Conversely, the E2F-4 antiserum clearly detected two closely migrating protein species that were present in the p107 immunoprecipitate. A very faint signal was detected when the E2F-4 antiserum was used to reimmunoprecipitate proteins associated with pRb (Fig. 5, lane 4). This could indicate that the E2F-4 antiserum shows a weak cross-reactivity with other E2Fs. Alternatively, some (<5%) E2F-4 may be bound to pRb.

We generated monoclonal antibodies that specifically recognize E2F-4 and not E2F-1, which recognize the same bands as detected by the polyclonal E2F-4 serum in ML-1 cells, HeLa cells, and BJAB cells (data not shown). Taken together, these data strongly suggest that E2F-4 interacts preferentially with p107, rather than with pRb.

E2F-4 is a phosphoprotein

The E2F-4 monoclonal antibodies recognize a 32P-labeled protein in a p107 immunoprecipitate indicating that E2F-4 is phosphorylated in vivo (Fig. 5). The finding that the E2F-4 antibodies recognized multiple closely migrating protein species (Fig. 5) could indicate that different proteins are recognized by the same antibody or...
that E2F-4 protein is subject to post-translational modifications that result in altered mobility in an SDS gel. To further investigate the nature of the different E2F-4 protein species that were present in p107 immunoprecipitates, we labeled ML-1 cells with [35S]methionine. mAb RK13 recognizes three protein species in a p107 immunoprecipitate (data not shown). The same pattern of bands was also detected when U2-OS cells were transfected with an E2F-4 expression vector (Fig. 6), indicating that the expression vector encodes the full-length E2F-4 protein. To investigate whether these protein species were all derived from the same protein by differential phosphorylation, we performed the following experiment. U2-OS cells were transfected with E2F-4, DP-1, and p107 expression vectors, labeled with [35S]methionine, and immunoprecipitated under low stringency conditions with p107 antibody. The p107 immunoprecipitate was treated in the presence or absence of calf intestine phosphatase, and p107-associated proteins were reimmunoprecipitated with mAb E2F-4. Figure 6 shows that following phosphatase treatment the three protein species that bind to p107 and are recognized by mAb RK13 were reduced to mostly a single fast migrating protein species, although some slower migrating species remain. Taken together, our data suggest that E2F-4 is a phosphoprotein and that the different E2F-4 protein species arise by differential phosphorylation of E2F-4.

Association of p107 with E2F-4 requires dimerization with DP-1

To investigate whether E2F-4 requires DP-1 for interaction with p107, we transfected U2-OS cells with p107 and E2F-4 in the absence or presence of an hemagglutinin (HA) epitope-tagged DP-1 expression vector. Transfected cells were labeled with [32P]orthophosphate and cell lysates were immunoprecipitated at low stringency with p107 antibody to allow coimmunoprecipitation of p107-associated proteins. The E2F-4 and DP-1 proteins were equally expressed in all transfections (data not shown). The anti-p107 immunoprecipitates were then boiled in 2% SDS and 15 mM DTT to release associated proteins and released proteins were reimmunoprecipitated with the anti-E2F-4 mAb RK13 or with mAb 12CA5 directed against the HA-tagged DP-1. The transfection of p107 with DP-1 did not result in complex formation between these two proteins (Fig. 7, lane 3). The same result was obtained when p107 was cotransfected with E2F-4 (Fig. 7, lane 5). In contrast, in cells transfected with all three expression vectors, p107, E2F-4, and DP-1, both E2F-4 and DP-1 were found in complex with p107 (Fig. 7, lanes 8,9). These results show that dimerization with DP-1 is not only required for efficient DNA binding by E2F-4, but is also essential for its interaction with p107.

Figure 6. E2F-4 is differentially phosphorylated. U2-OS cells were transfected with 10 μg pCMV–E2F-4 and 10 μg of pCMV–HA–DP-1. Cells were labeled with [35S]methionine, and cell lysates were subjected to sequential immunoprecipitation. First, lysates were immunoprecipitated with mAb 12CA5 directed against the HA-tagged DP-1. The immunoprecipitated proteins were treated in the absence or presence of calf intestine alkaline phosphatase. The proteins were then released as described in Material and methods and subjected to immunoprecipitation with anti-E2F-4 mAb RK13. Immunocomplexes were separated on 7.5% SDS–polyacrylamide gels and subjected to fluorography.

| Transfected | p107 | p107 | p107 | p107 |
|-------------|------|------|------|------|
| 1st antibody | p107 | p107 | p107 | p107 |
| 2nd antibody | N | RK13 | 12CA5 | N | RK13 | 12CA5 | N | RK13 | 12CA5 |
| MW (kD) | - | 97 | - | 69 | - | 46 |
Stimulation of cell proliferation by the E2F-4/DP-1 dimer

A number of cellular genes whose protein products are required for DNA synthesis contain E2F sites in their promoters [Nevins 1992]. Transcriptional activation of these genes by E2F is believed to be an important and, in most cases, essential step in the controlled expression of these genes during the cell cycle. It is expected, therefore, that deregulated expression of active E2F would stimulate cell proliferation. Consistent with this, it has been shown that E2F-1 can stimulate quiescent cells to initiate DNA synthesis [Johnson et al. 1993].

In this study we investigated the growth-promoting effects of E2F-4 and its dimerization partner DP-1 in cycling cells. Osteosarcoma cell line SaoS-2 was transiently transfected with DP-1 or E2F-4 or cotransfected with E2F-4 and DP-1 expression vectors. Three days later the cells were harvested and processed for flow cytometry analysis to determine the cell cycle status of the population. Transfected cells were identified by the cotransfected cell surface marker CD20 and analyzed in comparison to the control vector-transfected cells [Zhu et al. 1993]. Table 1 shows that overexpression of DP-1-stimulated cell cycle progression of the transfected population, decreasing the G1 population and increasing the S-phase population at the same time by almost 20%. Overexpression of E2F-4 alone also resulted in an increase in S-phase cells, although to a smaller extent. Overexpression of E2F-4 together with DP-1 exhibited the most dramatic effect by decreasing the G1 population and increasing the G2 + M-phase population as indicated in three independent experiments. These results indicate either that overexpression of E2F-4 and DP-1 promote entry into S phase or delay exit from later stages of the cell cycle. Because E2F-4 can act as a dominant oncogene in transformation of primary rodent fibroblasts [see below] we favor the explanation that E2F-4 promotes cell cycle progression rather than inhibiting exit from S phase and G2/M phase of the cell cycle.

Table 1. Overexpression of E2F-4/DP-1 stimulates cell proliferation

| Experiment | G0/G1 | S   | G2/M |
|------------|-------|-----|------|
| pCMV       | 51.1  | 31.7| 17.2 |
| pCMV–DP-1  | 44.6  | 33.3| 22.1 |
| pCMV–E2F-4 | 55.3  | 23.9| 20.7 |
| pCMV–E2F-4 + | 33.4 | 48.5| 18.1 |
| pCMV–DP-1  | 41.5  | 44.1| 14.4 |
| pCMV–E2F-4 | 28.5  | 43.1| 28.4 |
| pCMV–E2F-4 | 25.4  | 40.6| 34.0 |
| pCMV–DP-1  | 30.3  | 40.6| 29.1 |

SaoS-2 cells were transfected with pCMV–CD20 CD20 cell surface protein in combination with expression vectors pCMV, pCMV–DP-1, pCMV–E2F-4, or pCMV–E2F-4 together with pCMV–DP-1. Transfected cells were analyzed by FACS and cell cycle profiles of CD20-positive cells was determined. The percentage of cells in G0/G1, S, and G2/M phase are depicted.

Overexpression of E2F-4 and DP-1 together with ras causes transformation

The effect of E2F-4 on cell cycle progression suggests a role for E2F-4 in normal cell cycle control. If this is the case, overexpression of E2F-4 might result in deregulated growth control. Proteins that promote transition from G1 to S phase of the cell cycle may have immortalizing activity on primary cultures of fibroblasts. For example, both adenovirus E1A and c-Myc promote S phase entry [Heikkila et al. 1987; Moran and Mathews 1987; Eilers et al. 1992] and have immortalizing activity when introduced in primary rat embryo fibroblast (REF) cultures [Land et al. 1983; Ruley 1983].

To study the effect of E2F-4 on primary cultures of REFs, we transfected REFs with E2F-4, DP-1 expression vectors alone, or cotransfected with E2F-4 and DP-1. In all experiments a plasmid encoding a mutant Ha-ras oncogene was cotransfected to obtain full oncogenic transformation [Land et al. 1983]. Because both the E2F and DP-1 expression vectors contain a neomycin resistance gene, colonies of transformed cells could be selected in the presence of G418. After 4 weeks G418-resistant colonies were detected only in the E2F-4-, DP-1-, and ras-transfected cells and not in the E2F-4- plus ras- or DP-1-plus ras-transfected cells. Figure 8 shows the morphology of two colonies of REFs, E4R1 and E4R2, transfected with E2F-4, DP-1 and ras, in comparison to the primary REF culture. Both transfectants have a transformed morphology. Consistent with their transformed morphology both transfectants were able to form colonies in soft agar and gave rise to tumors with short latency periods when 5 x 10⁶ cells were injected in nude mice [data not shown].

The expression of E2F-4 and DP-1 in the transformed REF cell lines was measured in two independent assays. First, the REF cell lines were labeled with ³²P orthophosphate and subjected to immunoprecipitation with mAb 12CA5 directed against the Ha-tagged DP-1. Figure 9 shows that both E2F-4- plus DP-1-transfected REF cell lines, E4R1 and E4R2, but not control adenovirus 5-transformed REFs, expressed the 55-kD Ha-tagged DP-1 protein [cf. lanes 1, 3, and 5]. In the former two lines, mAb 12CA5 also immunoprecipitated a 60-kD protein that could be reimmunoprecipitated with mAb E2F-4 [Fig. 9, lanes 7, 9]. These data indicate that the immortalized REF cell lines express both DP-1 and E2F-4.

To further analyze the E2F DNA-binding activity in these REFs, we made nuclear extracts of E4R2 and adenovirus 5-transformed REFs and used these in a gel retardation assay. The E2F-4 transfectant E4R2 showed a dramatic increase in total amount of E2F DNA-binding activity, which was completely supershifted by the addition of the E2F-4 antiserum [data not shown]. Furthermore, most of the E2F activity in the E4R2 was free E2F, whereas in the adenovirus-transfected line only slower migrating complexes were observed that were not affected by the E2F-4 antiserum. We conclude that overexpression of E2F-4 and DP-1 with an increased E2F-4 DNA-binding activity in REFs, in combination with an
activated ras oncogene, results in the generation of transformed cell lines that have oncogenic activity.

Discussion

We have isolated a fourth member of the E2F gene family. E2F-4 differs from the three known E2Fs in that E2F-4 associates in vivo preferentially with the pRb-reliably. E2F-4 differs from the three known E2Fs in that activated ras oncogene, results in the generation of transformed cell lines that have oncogenic activity.

Figure 8. E2F-4/DP-1 overexpression causes transformation. Overexpression of E2F-4 in combination with DP-1 and mutant ras results in the outgrowth of G418-resistant colonies having a transformed morphology. (A) Primary REFs; (B) REFs transformed by E2F-4, DP-1, and ras [E4R1]; (C) REFs transformed by E2F-4, DP-1, and ras [E4R2]. Magnification, 52 ×.

Figure 9. E2F-4 expression in transformed REFs. Adenovirus-transformed primary fibroblasts [Ad5] and E2F-4/DP-1 transformed primary fibroblasts [E4R1 and E4R2] were labeled with [32P]orthophosphate and subjected to sequential immunoprecipitation. First, the lysates were immunoprecipitated with mAb 12CA5 against HA-tagged DP-1 [lanes 1,3,5] or control antibody [lanes 2,4,6]. The associated proteins were released as described in Materials and methods and subjected to reimmunoprecipitation with E2F-4 mAb RK13 [lanes 7,9,11] or control antibody [lanes 8,10,12]. Immune complexes were separated on 7.5% SDS-polyacrylamide gels and detected by autoradiography. Equal amounts of [32P]-labeled cell lysates were used for immunoprecipitation in this experiment.
in part, by binding to and inactivating the c-Myc protein (Beijersbergen et al. 1994). Our present data add E2F-4 to the short list of p107-interacting cellular proteins, and a striking similarity between c-Myc and E2F-4 becomes apparent from the present study. First, both c-Myc and E2F-4 appear to have a trans-activation domain that can bind p107, resulting in inhibition of trans-activation. Furthermore, our data indicate that E2F-4, like c-Myc, may promote progression from G1 to S phase of the cell cycle (Table 1; Heikila et al. 1987; Eilers et al. 1991). This interpretation is substantiated by the observation that E2F-4, like c-Myc, can cooperate with an activated ras oncogene in the transformation of REF cultures (Fig 8). Therefore, it will be worthwhile to study the possible involvement of E2F-4 in human cancer.

Although p107 can inhibit the activity of cell cycle regulatory proteins such as c-Myc and E2F-4, mutant forms of p107 do not appear to occur in human cancers. One possibility is that p107 is functionally redundant. The p107-related protein p130, which was recently isolated by molecular cloning, is a candidate for a protein having p107-like activity. p130 is structurally more related to p107 than to pRb (Hannon et al. 1993; Li et al. 1993b). p130 also has been observed in complexes with E2F DNA-binding activity and higher order complexes between p130 and cyclin E/cdk2 and cyclin A/cdk2 have also been observed (Cobrinik et al. 1993). However, p130/E2F complexes occur in different stages of the cell cycle as compared with the p107/E2F complexes. We are currently investigating whether E2F-4 can also associate with p130; however it is also possible that p130 interacts with yet other members of the E2F gene family. We have recently isolated a fifth member of the E2F gene family (M. Hijmans, M. Voorhoeve, and R. Bernards, unpubl.).

An important question that remains to be resolved concerns the regulation of E2F-4 activity in the cell cycle. From the study of the E2F-1–pRb interaction it is clear that not only phosphorylation of pRb but probably also of E2F-1 and DP-1 by cdks is important in controlling activity (Krek et al. 1994). In contrast, very little is known about the regulation of p107 by phosphorylation. That cdks play a key role in regulating p107/E2F complex formation is, however, quite likely.

Lees et al. (1992) have detected cell-cycle-regulated complex formation among E2F, p107, and cyclin E/cdk2 in the G1 phase of the cell cycle. In S phase, a complex of E2F, p107, and cyclin A/cdk2 was detected. The appearance of the different cyclin/cdk complexes in the E2F/p107 complex follows the kinetics of the synthesis of these cyclins. The role of these two cyclin/cdk complexes in controlling the E2F/p107 interaction is presently not known. It is possible that these kinases phosphorylate p107 and/or E2F-4. Our finding that E2F-4 is a phosphoprotein is consistent with a role for cyclin/cdk complexes in controlling E2F-4 activity. Whatever the targets of these cyclin/cdk complexes may be, their presence does not appear to lead to the dissociation of the p107/E2F complex. Because complexes between E2F and p107 are found throughout the cell cycle, it is not obvious when p107-interacting E2Fs are free to activate transcription of their target genes. We are currently studying the effect of the various cyclin/cdk complexes on E2F-4/p107 complex formation and the effect of these kinases on E2F-4 activity. The availability of the first p107-specific E2F should be a helpful tool in understanding the regulation of p107 by cdks and the differences between regulation of pRb and p107 by these kinases.

**Material and methods**

**Screening of cDNA libraries with p107**

The p107 protein probe used in the screening of cDNA expression libraries was GST–2TK–p107, containing the pocket region of human p107. The GST–2TK vector contains a consensus phosphorylation site for protein kinase A (Blanar and Rutter 1992). GST–p107 fusion protein was made in *Escherichia coli*, purified and labeled in vitro with [γ-32P]ATP as described to a sp. act. of >1×10⁵ cpm/µg of protein. A mouse embryo cDNA library in λXEX/lox (obtained from Novagen, Madison, WI) was screened with the p107 protein probe as described (Ayer et al. 1993). In a screening of 5×10⁹ phages, 3 positive phages were identified.

The partial mouse E2F-4 cDNA was used to screen additional mouse and human cDNA libraries. The human E2F-4 cDNA described here was isolated from the T84 colon carcinoma library (obtained from Stratagene).

**Plasmids**

pGST-E2F4 was constructed by cloning a fragment of the human E2F-4 cDNA encoding amino acids 108–300 in pGEX3X. For transfection experiments the following plasmids were used: pCMV E2F-4 was constructed by linking the 5′ end of mouse E2F-4 (encoding amino acid 1–16) to a fragment of the human E2F-4 cDNA encoding amino acids 17–413 at the conserved Nael site in pRC/CMV (Invitrogen). pCMV–HA–DP-1 has been described (Helen et al. 1993b). pCMV–E2F-1 was generated by inserting the coding sequence of pSP72 RBAP-1 (Kaelin et al. 1992) in pRC/CMV. The plasmids pCMV–pRb, pCMV–p107, pCMV–p107DE were described previously (Zhu et al. 1993).

**Cell lines**

Human C33A, ML-1, and U2-OS were cultured in Dulbecco's modified Eagle medium [DMEM] with 10% fetal calf serum (FCS). REFs were isolated from 13 day-old rat embryos and cultured in DMEM in the presence of 10% FCS. Transfections were performed using the calcium phosphate precipitation technique (Van de Eb and Graham 1980). Stable transfectants were obtained after selection with 0.5 mg/ml of G418 for 4 weeks.

**Immunological reagents**

To generate antibodies against E2F-4, a bacterial expression plasmid containing glutathione S-transferase (GST)–E2F–4 (amino acids 108–300) was generated (see above). GST–E2F-4 protein was made in *E. coli*, purified over glutathione–Sepharose beads, and used to immunize mice. After three rounds of immunization with GST–E2F-4, polyclonal serum was obtained. Monoclonal antibodies were prepared by fusion of splenocytes of immune BALB/c mice to Sp2/0 myeloma cells four days after the final boost. Positive tissue culture supernatants were identified in an ELISA. Supernatants of 19 different ELISA-positive hybridoma cell lines were tested in immunoprecipitation. For the ex-
periments described in this paper we used the polyclonal serum pc11 and the mAb RK13. Antibodies against p107 (SD2, SD4, SD9, SD6, SD15), pRb (XZ77 and C36) and against the HA epitope (12CA5) have been described previously (Hu et al. 1991; Zhu et al. 1993).

**Metabolic labeling**

Cells were incubated for 1 hr in phosphate free DMEM or methionine-free DMEM in the presence of 10% FCS, followed by an incubation in medium containing 5 mCi [32P]orthophosphate or 250 μCi Trans [35S]label for 4 hr.

**Immunoprecipitations**

Cells were lysed in ELB + buffer containing 5 mm EDTA, 1 mm sodium orthovanadate, 0.2 mm sodium pyrophosphate, 1 μg/ml chymostatin and aprotinin, and 1 mm phenylmethylsulfonyl fluoride (PMSF) and immunoprecipitated as described previously [Beijersbergen et al. 1994].

**Phosphatase treatment**

Transfected U2-OS cells were labeled with [35S]methionine and precipitated with mAb 12CA5 as described. After the first immunoprecipitation, the precipitated proteins were washed three times in ELB + buffer followed by a wash with 1 ml of phosphatase buffer [50 mm Tris-HCl (pH 9.0), 5 mm MgCl2, containing 1 mm PMSF, and 1 μg/ml of both chymostatin and aprotinin]. The immunoprecipitated proteins were split into two and resuspended in 50 μl of phosphatase buffer and incubated for 30 min at 30°C in the absence or presence of 5 units of calf intestine alkaline phosphatase (Boehringer). Reactions were terminated by addition of 50 μl of 2× releasing buffer [ELB + containing 4% SDS and 30 mM DTT] and boiled for 10 min. After this, protein A-Sepharose beads were removed by centrifugation and the supernatant diluted to 1 ml in ELB buffer and precleared with protein A-Sepharose beads. The released proteins were then immunoprecipitated with mAb RK13. The immunoprecipitated proteins were collected in PBS. After centrifugation at 1000g, the cells were collected in PBS. After centrifugation at 1000g, the cells were resuspended in 100 μl of 0.1 M Tris-HCl (pH 8.0). The cells were freeze-thawed three times and centrifuged at 15,000g at 4°C for 10 min. Supernatants were assayed for luciferase activity [Promega, Luciferase system] and CAT activity using the phase extraction assay [Seed and Sheen 1988].

**Gel retardation assays**

Transfected U2-OS cells were washed three times with PBS, and the cells were collected in PBS. After centrifugation at 1000g, the cells were resuspended in 100 μl of 5× binding buffer [100 mm Hepes [pH 7.4], 0.5 μm KCl, 5 mm MgCl2, 0.5 mm EDTA, 35% glycerol, 5 mm NaF]. After one freeze-thaw step, the cells were kept on ice for 30 min. The extracts were centrifuged at 100,000g for 30 min at 4°C. The supernatant was used in gel retardation assays.

Gel retardation assays for transiently transfected U2-OS cells were performed as described previously [Helin et al. 1993b] with minor modifications. Ten micrograms of cell extract was used in 20-μl reactions containing 1× binding buffer and 1 μg of sonicated salmon sperm DNA. Reactions were incubated for 10 min at room temperature, after which 0.5 ng of 32P-labeled oligonucleotide containing the consensus E2F DNA-binding site (Shanacruz) was added and the reaction was incubated for additional 20 min at room temperature. The reaction products were separated on a 3.5% polyacrylamide gel in 0.25× TBE at 90 V for 2.5 hr. The gel was then dried, and reaction products were visualized by autoradiography.

**Acknowledgments**

We thank Drs. Kristian Helin, William Kaelin, Marc Ewen, and Brian Dynlacht for the kind gift of various plasmids, Nicholas Dyson for p107 antibodies, Jos Jonkers for the kind gift of a mouse tissue RNA blot, and Jacky Lees for helpful discussions. We thank Nancy Walworth for helpful discussions and critical reading of the manuscript. This work was supported by a grant from the Netherlands Society for Scientific Research (NWO). L.Z. was supported by the Leukemia Society of America.

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Development of several organs that require inductive epithelial–mesenchymal interactions is impaired in LEF-1-deficient mice

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Lymphoid enhancer factor 1 (LEF-1) is a sequence-specific DNA-binding protein that is expressed in pre-B and T lymphocytes of adult mice, and in the neural crest, mesencephalon, tooth germs, whisker follicles, and other sites during embryogenesis. We have generated mice carrying a homozygous germ-line mutation in the LEF-1 gene that eliminates its protein expression and causes postnatal lethality. The mutant mice lack teeth, mammary glands, whiskers, and hair but show no obvious defects in lymphoid cell populations at birth. The LEF-/-deficient mice also lack the mesencephalic nucleus of the trigeminal nerve, the only neural crest-derived neurons normally present within the brain, but no deficiency can be detected in other neural crest-derived neuronal populations. Together, the pattern of these defects suggest an essential role for LEF-1 in the formation of several organs and structures that require inductive tissue interactions.

[Key Words: Lymphoid enhancer factor 1; neural crest; organogenesis; mouse development]

Received August 26, 1994; revised version accepted September 26, 1994.

Naturally occurring and experimentally induced mutations have revealed that many developmental processes are controlled by transcriptional regulatory proteins. In particular, DNA-binding proteins that are expressed in specific cell lineages, or at specific stages of differentiation, participate in the developmental regulation of gene expression (for review, see Gruss and Walther 1992; Voss and Rosenfeld 1992; Weintraub 1993). Although some transcriptional regulators are restricted to single cell lineages, most are expressed in multiple lineages and often by phenotypically unrelated cell types. Such pleiotropic expression may indicate that a transcriptional regulator can subserve multiple functions through combinatorial association with distinct proteins in different cell types (Herskowitz 1989). Alternatively, pleiotropic expression may suggest that a given factor participates in a developmental process that is common to many different cell types or tissues.

Lymphoid enhancer-binding factor 1 (LEF-1) was cloned initially as a pre-B and T lymphoid-specific gene encoding a DNA-binding protein of the family of high mobility group (HMG) proteins (Travis et al. 1991; Waterman et al. 1991). LEF-1 is encoded by a gene located on chromosome 3 in the mouse and chromosome 4 in humans (Milatovich et al. 1991). Sequence-specific recognition of DNA by LEF-1 protein was found to be governed by an 85-amino-acid region, termed the HMG domain, that displays sequence homology with other members of this family of proteins (Giese et al. 1991). Functional and biochemical characterization of LEF-1 indicated that this protein participates in the regulation of the enhancer associated with the T-cell receptor (TCR)α gene (Travis et al. 1991; Waterman et al. 1991). LEF-1 protein has the capacity to induce a sharp bend in the DNA helix and is dependent on other enhancer-bound proteins to activate transcription (Giese et al. 1992). Together with the requirement for a particular arrangement of factor-binding sites in the TCRα enhancer, these observations were interpreted to suggest an "architectural" role for LEF-1 in the assembly of a higher order nucleoprotein complex (Grosschedl et al. 1994; Tjian and Maniatis 1994). In addition, LEF-1 contains a transactivation activation domain that is enhancer context specific and may mediate, directly or indirectly, the association with other enhancer-bound proteins (Carlsson et al. 1993, Giese and Grosschedl 1993).

Despite these insights into the biochemical properties of LEF-1, its biological role is still obscure. In the adult mouse, the LEF-1 gene is expressed specifically in lymphoid tissues (Travis et al. 1991; Waterman et al. 1991). During mouse embryogenesis, however, a wider pattern of RNA expression from the LEF-1 gene was detected by in situ hybridization analysis (Oosterwegel et al. 1993). At day 10.5 of embryogenesis (E10.5), LEF-1 is expressed
Regulation of the retinoblastoma protein-related p107 by G₁ cyclin complexes

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The orderly progression through the cell cycle is mediated by the sequential activation of several cyclin/cyclin-dependent kinase (cdk) complexes. These kinases phosphorylate a number of cellular substrates, among which is the product of the retinoblastoma gene, pRb. Phosphorylation of pRb in late G₁ causes the release of the transcription factor E2F from pRb, resulting in the transcriptional activation of E2F-responsive genes. We show here that phosphorylation of the pRb-related p107 is also cell cycle regulated. p107 is first phosphorylated at 8 hr following serum stimulation of quiescent fibroblasts, which coincides with an increase in cyclin D1 protein levels. Consistent with this, we show that a cyclin D1/cdk4 complex, but not a cyclin E/cdk2 complex, can phosphorylate p107 in vivo. Furthermore, phosphorylation of p107 can be abolished by the overexpression of a dominant-negative form of cdk4. Phosphorylation of p107 results in the loss of the ability to associate with E2F-4, a transcription factor with growth-promoting and oncogenic activity. A p107-induced cell cycle block can be released by cyclin D1/cdk4 but not by cyclin E/cdk2. These data indicate that the activity of p107 is regulated by phosphorylation through D-type cyclins.

[Key Words: Cell cycle; cyclins; cyclin-dependent kinase; E2F; p107]

Received February 6, 1995; revised version accepted April 25, 1995.
is whether the cyclin D1-associated kinase activity varies during the cell cycle.

The product of the retinoblastoma gene, pRb, is a substrate for G1 cyclin/cyclin-dependant kinase (cdk) complexes. Extracts from insect cells infected with recombinant baculoviruses that express either cyclin E and cdk2 or D-type cyclins and cdk4 efficiently phosphorylate pRb in vitro [Ewen et al. 1993; Kato et al. 1993]. In human osteosarcoma cells, expression of cyclin E, D2, and D3, but not D1, resulted in hyperphosphorylation of pRb [Hinds et al. 1992, Ewen et al. 1993]. Significantly, a pRb-induced cell cycle block could be released by ectopic expression of cyclin E, D2, and D3, but not effectively by cyclin D1 expression [Hinds et al. 1992, Ewen et al. 1993].

The retinoblastoma protein and the related p107 and p130 interact with several cellular polypeptides including E2F, a transcription factor that controls gene expression during the cell cycle [Cao et al. 1992; Cobrinik et al. 1993]. E2F DNA-binding activity consists of a heterodimeric complex containing an E2F component complexed to a DP component [Bandara et al. 1993; Helin et al. 1993; Krek et al. 1993]. The E2F component of the heterodimer is encoded by at least five closely related polypeptides. E2F-1, E2F-2, and E2F-3 associate in vivo only with pRb and not with the related p107 [Lees et al. 1993]. E2F-4, in contrast, interacts with p107 and p130 but not with pRb [Beijersbergen et al. 1994b; Ginsberg et al. 1994, Vairo et al. 1995, R.L. Beijersbergen and R. Bernards, unpubl.], whereas E2F-5 interacts preferentially with p130 [Hijmans et al. 1995]. E2F binds preferentially to hypophosphorylated pRb, suggesting that complex formation between pRb and E2F is regulated by phosphorylation of pRb by cyclin/cdk complexes [Buchkovich et al. 1989; Chen et al. 1989; DeCaprio et al. 1989; Chellappan et al. 1991]. Hypophosphorylated pRb is found mostly in the G1 phase of the cell cycle, whereas the hyperphosphorylated form of pRb is first observed at the G1 to S transition [Buchkovich et al. 1989; Chen et al. 1989; DeCaprio et al. 1989].

The complexes between E2F and p107 show a more complex pattern of appearance during the cell cycle. In late G1, DNA-binding complexes have been observed that contain E2F, p107, cyclin E, and cdk2. In S phase cyclin E is no longer found in these complexes; instead, E2F is found associated with p107, cyclin A, and cdk2 [Lees et al. 1992, Shirodkar et al. 1992]. In spite of the presence of cyclin/cdk complexes in association with p107, very little is known about the regulation of p107 by phosphorylation. In contrast to pRb, cell cycle-regulated phosphorylation of p107 has not been observed. We show here that the growth inhibitory activity of p107 is subject to regulation by phosphorylation by cyclin/cdk complexes.

Results

**Effect of cyclin E on p107**

In late G1 phase of the cell cycle, p107 is found in a multiprotein complex that contains E2F DNA-binding activity, cyclin E, and cdk2 [Lees et al. 1992]. To study the effect of cyclin E and its associated kinase cdk2 on the E2F/p107 complex, we used a transient transfection assay with E2F-4. We have shown recently that E2F-4 associates in vivo specifically with p107 and not with pRb. As a control, we studied the effect of cyclin E/cdk2 on the pRb/E2F-1 complex. We cotransfected a chloramphenicol acetyltransferase (CAT) reporter gene harboring upstream E2F sites with either E2F-4 and DP-1 expression vectors or with E2F-1 and DP-1 expression vectors. Figure 1, A and B, shows that both the E2F-4/DP-1 and the E2F-1/DP-1 heterodimers efficiently activated the CAT reporter gene (tracks 3). As expected, cotransfection of p107 inhibited E2F-4 trans-activation and pRb expression suppressed E2F-1 trans-activation (tracks 5). When cyclin E/cdk2 and pRb expression vectors were cotransfected, a release of E2F-1 inhibition by pRb was observed, presumably as a result of phosphorylation of pRb by cyclin E/cdk2 [Fig. 1A, track 6] [Hinds et al. 1992, Ewen et al. 1993]. Surprisingly, cotransfection of cyclin E/cdk2 with p107 failed to relieve p107 inhibition of E2F-4 [Fig. 1B, track 6]. We conclude that cyclin E/cdk2 acts differently on pRb and p107 in that cyclin E/cdk2 releases pRb, but not p107 inhibition of E2F.

**Effect of D-type cyclins on p107**

Next, we studied the effect of cyclin D1 on p107. Figure 2 shows that in contrast to cyclin E/cdk2, cyclin D1, together with its major catalytic partner cdk4, can release p107 inhibition of E2F-4 and pRb inhibition of E2F-1 [Fig. 2A,B, cf. tracks 4 and 7]. The inactivation of p107 as an inhibitor of E2F-4 activity could be caused by several mechanisms. First, cyclin D1/cdk4 could phosphorylate p107, thereby releasing active E2F-4. An alternative mechanism of cyclin D1 action could be the direct binding of cyclin D1 to the pocket region of p107, as cyclin D1 shares a motif (LXCXE) with a number of viral transforming proteins that bind avidly to p107 and pRb through this motif [Dowdy et al. 1993]. However, neither cyclin D1 alone nor cdk4 alone was able to release p107 and pRb inhibition of E2F activity [Fig. 2A,B]. This suggests that a cyclin D/cdk complex is required to release p107 inhibition of E2F-4.

To study the kinase requirement of D-type cyclins to inactivate p107, we cotransfected E2F-4 and p107 with other combinations of D-type cyclins and cdks. To address the specificity of the kinase, we expressed cyclin D3, instead of cyclin D1, in combination with either cdk2 or cdk4. Both cdk2 and cdk4, when bound to cyclin D3, have significant kinase activity toward pRb, whereas cyclin D1 only forms an active complex with cdk4 [Ewen et al. 1993]. Figure 3 shows that although cyclin D1/cdk4 and cyclin D3/cdk4 effectively rescued E2F-4 inhibition by p107, no release of inhibition was observed with cyclin D3/cdk2 [Fig. 3, cf. tracks 6, 8, and 9]. These data indicate that not only the cyclin, but also the associated kinase contributes to the activity of the cyclin/cdk complex toward p107.

The data shown above are consistent with a model in