E2F-4, a new member of the E2F transcription factor family, interacts with p107

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The E2F family of transcription factors has been implicated in the regulation of cell proliferation, and E2F-binding sites are present in the promoters of several growth-regulating genes. E2F family members are functionally regulated, in part, by complex formation with one or more members of the nuclear pocket protein family, RB, p107, and p130. Pocket protein regulation of E2F likely contributes to normal cellular growth control. While the three cloned species of E2F, E2F-1, E2F-2, and E2F-3, are known to be targets of RB interaction, no E2F species has yet been shown to be a specific p107 or p130 target. Here, we describe the cloning of a new member of the E2F family, E2F-4, which forms heterodimers with a member(s) of the DP family and, unlike some family members, is present throughout the cell cycle and appears to be a differentially phosphorylated p107-binding partner. p107 binding not only can be linked to the regulation of E2F-4 transcriptional activity, but also to suppression of the ability of E2F-4 to transform an immortalized rodent cell line.

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The transcription factor E2F was originally identified as a cellular DNA-binding protein required for activation of the adenovirus E2A promoter [Kovesdi et al. 1986; Yee et al. 1987]. The interaction of E2F with the sequence TTTCGCG, present twice in the E2 promoter, stimulates its transcription [Loeken and Brady 1989; Yee et al. 1989]. E2F sites were subsequently detected in the promoters of multiple growth-responsive and growth-promoting cellular genes, including *c-myc*, *c-myb*, *N-myc*, dihydrofolate reductase (DHFR), thymidine kinase (TK), thymidine synthetase, DNA polymerase α , cyclin A, cyclin D1, and *cdc2* [Blake and Azizkhan 1989; Hiebert et al. 1989, 1991; Thalmeier et al. 1989; Mudryj et al. 1990; Pearson et al. 1991; Dalton 1992; Nevins 1992; Herber et al. 1994; Phillip et al. 1994; Yamamoto et al. 1994]. The E2F-binding site was shown to be involved in the activation of the *c-myc*, DHFR, TK, *B-myb*, and *cdc2* genes following serum stimulation [Blake and Azizkhan 1989; Mudryj et al. 1990; Dalton 1992; Means et al. 1992; Lam and Watson 1993]. These observations suggest that E2F activity plays a role in fostering cell-cycle progression.

Regulation of E2F activity is achieved, in part, through its interaction with certain cellular proteins. The retinoblastoma susceptibility gene product (RB) binds directly

to certain E2F species and inhibits their ability to *trans*-activate [Bagchi et al. 1991; Chellappan et al. 1991; Chittenden et al. 1991; Dalton, 1992; Hamel et al. 1992; Hiebert et al. 1992; Weintraub et al. 1992; Zamanian and La Thangue 1992]. Furthermore, RB growth suppression activity in some assays correlates with its ability to interact with E2F [Qin et al. 1992]. The RB-related protein, p107, forms two independent complexes with E2F. In one of them, cyclin A and cdk2 are also present, while cyclin E and cdk2 are present in the other [Cao et al. 1992; Devoto et al. 1992; Pagano et al. 1992; Shirodkar et al. 1992]. E2F and a third pocket protein, p130, form a complex analogous to the p107-E2F complex(es) [Cobrinik et al. 1993]. Where tested, pocket protein-binding results in suppression of the *trans*-activation function of an E2F target species. Individual pocket protein-E2F complexes appear and disappear at different times in the cell cycle [Shirodkar et al. 1992], and the available evidence suggests a model in which each has a specific set of transcriptional consequences that contribute to the regulation of cell cycle arrest/progression. Moreover, all of these pocket protein E2F-containing complexes can be dissociated by certain small DNA tumor virus oncoproteins, including adenovirus E1A, papovavirus large T antigen, and human papilloma virus E7 [for review, see La Thangue 1994], leading to the release of free E2F, which is believed to be the E2F species that *trans*-activates.

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but not identical, to those present in the DNA-binding domain of E2F-1, E2F-2, E2F-3.

This product was then used as a probe to screen λ libraries prepared from HeLa (a human cervical carcinoma-derived cell line) and Nalm6 cells (a human pre-B leukemic cell line). One million plaques from each library were screened under high stringency conditions. Following plaque purification and *in vivo* excision, six positive cDNA molecules were isolated, five from the HeLa library and one from the Nalm6 library. Sequencing of the positive clones revealed a colinear sequence of 2100 nucleotides that contained an open reading frame (ORF), open at its 5' end. From the imputed protein sequence beginning at the first methionine, a protein product of 411–416 amino acids was predicted (Fig. 1A). The reason for the variation in protein size will be discussed below. This ORF, present in three of these cDNA products, appears to encode a new, full-length E2F species, which we have termed E2F-4.

E2F-4 structural features

The overall identity between E2F-4 and E2F-1 is 27% at the amino acid sequence level. Not surprisingly, given the high degree of overall homology among all previously known E2F species (Lees et al. 1993), a significant degree of homology to E2F-2 and E2F-3 was also detected. The homology between E2F-4 and the other three proteins is especially evident in four subsegments of its sequence.

The first spans residues 12–81 in E2F-4. This region corresponds to a segment of the E2F-1 DNA-binding domain, residues 102–196 of E2F-1 (Helin et al. 1992). The overall homology between E2F-1 and E2F-4 is 67% in this region, although it is more pronounced in the carboxy-terminal part of this segment—89% over the 28 residues noted in Figure 1B, box H2. In contrast, there was only 50% homology in the amino-terminal 42 amino acids of this region (Fig. 1B, box H1).

Similar overall degrees of homology exist between E2F-4 and E2F-2, as well as E2F-4 and E2F-3, in the putative E2F-4 DNA-binding domain (Fig. 1B, boxes H1 + H2). A second region of E2F-4 homology to the three previously known E2F species consists of a hydrophobic heptad repeat unit of 29 residues located immediately carboxy-terminal to the putative DNA-binding domain (Fig. 1B, box Z). As in the other E2F species, five hydrophobic residues, present at every seventh position in this segment, were detected. The intervening residues are mostly not conserved among the four E2F proteins that were analyzed (Fig. 1; Lees et al. 1993).

A third region of E2F-1, E2F-2, and E2F-3 homology (53% homology among these proteins) has been termed the “marked box” (Lees et al. 1993). It spans residues 251–317 in E2F-1 and is of unknown function (Fig. 1B, MB). In comparison, E2F-4 revealed limited homology to the others in this region. In comparison with E2F-1, for example, it was only detected within stretches of 5 and 15 residues located at the amino and carboxyl termini of the MB segment, respectively.

The fourth region of homology to E2F-1, E2F-2, and E2F-3 is located at its carboxyl terminus. In that region, E2F-4 contains a stretch of 18 residues that are 55% homologous to the analogous sequence of E2F-1. Similar homology was noted to the analogous sequences of E2F-2 and E2F-3 (50% and 55%, respectively). This short sequence constitutes the RB-binding domain of E2F-1 (Helin et al. 1992) and is likely the pocket protein-binding motif of E2F-2 and E2F-3, as well.

The new E2F species is also characterized by certain unique structural features. First, in E2F-1, E2F-2, and E2F-3 the DNA-binding domain is preceded by 121, 130, and 178 amino-terminal residues, respectively. This portion of these three proteins also contains two conserved regions. One is a stretch of basic amino acids that is adjacent to the DNA-binding domain, and the second, located more toward the amino terminus, is a sequence that mediates direct interaction with cyclin A in E2F-1 (Krek et al. 1994). In E2F-4, this entire region, including the cyclin-binding and the basic domains, is absent, and the DNA-binding domain begins at residue 13 of the protein sequence (Fig. 1B).

A second structural difference between E2F-4 and E2F-1, E2F-2, and E2F-3 is the spacing between the MB and the region containing the acidic *trans*-activation domain within which the pocket protein-binding domain is imbedded. In E2F-1, E2F-2, and E2F-3 this spacing is very similar, 78, 62, and 59 residues, respectively. In E2F-4 the space between these regions is significantly larger, 149 residues. This increased spacing is partially attributable to another unique feature of E2F-4, a repeat of the triplet, CAG, which encodes a polyserine array. Similar CNG repeats are present in a number of other genes, and changes in their size have been correlated with a number of human disorders (Morell 1993).

Polymorphic variations in the (CAG)_n trinucleotide repeat of E2F-4

The CAG repeat in the E2F-4-coding region varied in size in the few individual cDNAs isolated during the original cloning of E2F-4. Of the four cDNAs sequenced in this region, two contained 16 CAG repeats, and the other two contained 13 and 11 repeats, respectively. Because of this variability, the repeat length was measured in a group of DNA samples using PCR with oligonucleotides flanking the CAG repeat region. All 55 samples analyzed yielded a PCR product corresponding to a DNA molecule containing 13 CAG repeats. In 7 of these samples, there was an additional band corresponding to DNA molecules containing 7, 9, 11, 14, or 16 CAG repeats (data not shown). This result suggested that the individuals whose DNAs yielded these bands are heterozygotes for E2F-4, that is, they carry two alleles with different numbers of CAG repeats, and the E2F-4 reading frame in the (CAG)_n-encoded region is maintained in all cases. Because E2F-4 containing 11 or 16 serines in the polyserine array functioned identically in DNA binding and *trans*-activation assays (see Fig. 3A,B, below), the significance of the existing variations is, at the moment, unclear.

Expression and cell cycle analysis of E2F-4

A DNA fragment of E2F-4 cDNA was used to probe a Northern blot containing poly(A) RNA from various human tissues (Clontech). A complementary RNA species of ~2.2 kb was present in all tissues tested, including heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas (data not shown).

In an attempt to determine the pattern of E2F-4 mRNA expression during the cell cycle, NIH-3T3 fibroblasts were growth-arrested by serum deprivation and allowed to resume growth following serum readdition. Their cell cycle state was monitored by FACS. As noted in Figure 2, A and B, E2F-4 mRNA was present in the growth-arrested state, and its abundance did not change significantly as cells moved into and through the cell cycle. Similar results were obtained with primary human T cells (data not shown). This pattern of expression is significantly different from that of E2F-1 and DP-1 mRNA levels, which are cell-cycle controlled and are markedly up-regulated following serum stimulation (Fig. 2A; Kaelin et al. 1992; Slansky et al. 1993; Li et al. 1994). E2F-1 homologous RNA was detectable in G₀ cells, although the bands were not intense (Fig. 2A,B). The intensity of the two fastest migrating bands increased dramatically 8–12 hr after serum stimulation. The DP-1

mRNA level was readily detected in growth-arrested cells, although it, too, increased at the same time as E2F-1 (Fig. 2A,B). Western blot analysis, using protein extracts of NIH-3T3 cells, which were similarly growth arrested and serum induced, indicated that the E2F-4 protein was also present in the growth-arrested cells and that its levels did not change significantly as cells resume growth (Fig. 2C). Interestingly, E2F-4 migrated as a heterogeneous set of bands (~57–64 kD), the basis for which is discussed below.

E2F-4 binds to an E2F recognition site

The specific DNA-binding activity of the newly cloned protein was tested. Specifically, we synthesized a glutathione S-transferase GST–E2F-4 fusion protein and then measured its ability to bind to an oligonucleotide corresponding to the E2F-binding site within the DHFR promoter (Shirodkar et al. 1992). As can be seen in Figure 3A, the fusion protein demonstrated clear oligonucleotide-binding activity in a bandshift assay. A wild-type, but not a mutant E2F site-containing oligonucleotide competed with the labeled probe for binding to the protein, implying that the interaction observed was specific for an intact E2F site. E2F-4 species with 11 and 16

Figure 2. Cell cycle analysis of E2F-4 expression. (A) Cell cycle analysis of E2F-4 mRNA abundance. NIH-3T3 cells were starved for 65 hr in standard medium containing 0.5% BCS. Cells were then stimulated by the addition of 20% BCS and harvested at various times after serum addition for both FACS analysis and RNA extraction. Poly(A)⁺-enriched RNA was obtained, and 7 µg of each sample was subjected to Northern blot analysis using human E2F-4 cDNA as a probe. The Northern blot was then stripped and reprobed with cDNA of the acidic ribosomal phosphoprotein PO, 36B4, as a control for RNA loading, and with murine E2F-1 and murine DP-1 cDNAs. Time (in hours) after serum addition is indicated at the top. The cell cycle distribution of the cells under analysis is indicated at the bottom. (B) Densitometric analysis of the results depicted in A. Autoradiographs presented in A were quantitated by laser densitometry. The values obtained were normalized relative to the level of 36B4 gene expression and presented as the fold induction relative to the signal present at time zero (0). For E2F-1, where three bands were present (see text), the data reflect the major band corresponding in size to the previously reported murine E2F-1 mRNA (Li et al. 1994). (■) E2F-4; (×) E2F-1; (○) DP-1. (C) Cell cycle analysis of E2F-4 protein abundance. Whole-cell extracts were prepared from asynchronous cultures of human U937 cells and NIH-3T3 cells, that had been growth arrested and serum induced essentially as described in A. At the indicated times (hours) after serum addition, E2F-4 protein abundance was analyzed by Western blotting using anti-E2F-4 mAb GG22 as described in Materials and methods.

Figure 3. E2F-4 DNA binding and promoter *trans*-activation depend on the presence of an E2F site. (A) E2F-4 binds DNA containing an E2F site. Fifty nanograms of purified GST-E2F-4 fusion proteins were incubated with a labeled oligonucleotide corresponding to the E2F-binding site in the DHFR promoter (Shirodkar et al. 1992). A 100-fold excess of wild type (wt) or mutant (mut) competitor oligonucleotide was added, where indicated. The segments of E2F-4 cDNA used for the construction of the various GST-E2F-4 fusion proteins described here were those encoding residues 13–150 [GST-E2F4 (13–150)] or full-length E2F-4 containing either 11 [GST-E2F4(11)] or 16 [GST-E2F4(16)] serines in the polyserine region. (B) *Trans*-activation by E2F-4. U2OS cells were transfected with 4 μ g of expression plasmid, pCMV-E2F-4(11) or pCMV-E2F-4(16), 6 μ g of reporter plasmid, pE2wtCAT (wtE2), pE2(–64/–60, –45/–36)CAT (mE2F E2), or pE2(–80/–70)CAT (mATF E2), and 2 μ g of pCMV β GAL. Cell extracts were prepared 40 hr after transfection, and a CAT assay was performed on the relevant cell extracts, as described in Materials and methods. Amounts of extracts used for the CAT assay were normalized based on their β -galactosidase activity.

4(11) or pCMV-E2F-4(16), 6 μ g of reporter plasmid, pE2wtCAT (wtE2), pE2(–64/–60, –45/–36)CAT (mE2F E2), or pE2(–80/–70)CAT (mATF E2), and 2 μ g of pCMV β GAL. Cell extracts were prepared 40 hr after transfection, and a CAT assay was performed on the relevant cell extracts, as described in Materials and methods. Amounts of extracts used for the CAT assay were normalized based on their β -galactosidase activity.

serines present in the polyserine region displayed similar DNA-binding activity and specificity. A deleted version of E2F-4 containing only residues 13–150 also bound specifically to the E2F DNA, revealing that the E2F-4 DNA-binding domain is located within this region, as predicted earlier (see above).

E2F-4 *trans*-activates the adenovirus E2 promoter

E2F activity includes at least two components, binding to a specific DNA sequence and the ability to activate certain promoters containing this sequence. Therefore, we asked whether E2F-4 can *trans*-activate a promoter containing an E2F DNA-binding site(s). U2OS cells were transfected with an E2F-4 expression vector, pCMV-E2F-4, and a reporter plasmid containing E2F DNA-binding sites. Three such reporters were tested. Each contained the chloramphenicol acetyltransferase (CAT) gene linked to the wild-type adenovirus E2 promoter, to an E2 promoter lacking its activating transcription factor (ATF) site (–80/–70), or to a mutant E2 promoter lacking its two E2F sites (–64/–60, –45/–30; Loeken and

Brady 1989). Cotransfection of pCMV-E2F-4 and the wild-type E2 promoter led to a substantial increase in the level of CAT activity. E2F-4-dependent *trans*-activation was also observed in the absence of the ATF site. However, the protein failed to *trans*-activate the E2 promoter lacking the two E2F sites (Fig. 3B). These results imply that E2F-4 can *trans*-activate and that this function depends on the presence of an E2F site in the responding promoter. As was the case for the DNA-binding activity, E2F-4 containing either 11 or 16 serines *trans*-activated equally well in these assays, and the number of serines did not affect the E2F site requirement for this function. Similar results were obtained with an E2F-4 species containing 13 serines (data not shown). Hence, within certain limits, the extent of the polyserine run had no significant effect on the measured function of this protein.

E2F-4 can form heterodimers with DP-1

Because E2F-1 functions as a heterodimer with DP-1 (Bandara et al. 1993; Helin et al. 1993; Huber et al. 1993; Krek et al. 1993), the ability of the E2F-4 protein to in-

teract with DP-1 was assessed. U2OS cells were transfected with expression vectors encoding E2F-4, DP-1, hemagglutinin (HA)-E2F-4, and/or HA-DP-1. When extracts of the relevant cultures were employed in an E2F bandshift assay, a band (labeled A) which was present, but less intense, in untransfected cells and cells transfected with HA-DP-1, could be detected in the E2F-4 transfected cells (Fig. 4A, lane 4). Unlike the case of the other two cultures, this band in the HA-E2F-4 transfected culture was supershifted (to band B) by anti-HA antibody, 12CA5, indicating the presence of HA-E2F-4. This result again demonstrates the ability of E2F-4 to bind to an E2F site *in vivo*. As reported previously, cells transfected with HA-DP-1 alone displayed no additional bandshift activities (Fig. 4A, lanes 2,3), but when cells were cotransfected with E2F-4 and HA-DP-1 or HA-E2F-4 and DP-1, the newly detected E2F bandshift activity (band A), described above, was considerably more intense than that detected in cells transfected with HA-E2F-4 alone (Fig. 4A, cf. lanes 4, 6, and 8), and much of it was supershifted with HA antibody. When cells were cotransfected with untagged E2F-4 and DP-1, the same gel shift band was detected as in cells cotransfected with the HA-tagged versions of these proteins (Fig. 4A, cf. lanes 8 and 10), but it could not be supershifted by the anti-HA antibody 12CA5, demonstrating that the anti-HA antibody does not bind to either untagged proteins. Thus, under conditions in which both E2F-4 and DP-1 were overproduced, the two proteins interacted to form a prominent DNA-binding complex.

Cotransfection of E2F-4 and DP-1, together with an E2-CAT reporter plasmid, resulted in two- to threefold more CAT activity than was detected when E2F-4 was transfected alone (Fig. 4B). Similar results were obtained in different cell lines and with different promoters containing E2F DNA-binding sites (data not shown). Therefore, heterodimerization of E2F-4 and DP-1 leads to an increased *trans*-activation potential of E2F-4, just as was noted for E2F-1/DP-1 heterodimers (Bandara et al. 1993; Helin et al. 1993; Huber et al. 1993; Krek et al. 1993).

E2F-4 interacts with p107 *in vivo*

E2F bandshift activities exist in complexes containing RB, p107 and p130 (Shirodkar et al. 1992; Cobrinik et al. 1993). E2F-1, E2F-2, and E2F-3 appear to interact with RB but not with p107 *in vivo* (Lees et al. 1993). In an effort to determine the pocket protein-binding specificity of E2F-4, a whole-cell extract was prepared from U2OS cells transfected with HA-E2F-4. Half of the extract was immunoprecipitated with an anti-RB antibody (N9; Shirodkar et al. 1992), and the other half with an affinity-purified, polyclonal anti-p107 antibody (Shirodkar et al. 1992). Both precipitates were treated with deoxycholate (DOC) to release potentially associated proteins, and the treated supernatants were assayed for E2F DNA-binding activity. As predicted, E2F bandshift activity was detected in the supernatants derived from both the RB and p107 immunoprecipitates. Moreover, in both cases, ad-

Figure 4. Coexpression of E2F-4 and DP-1 cDNAs results in increased E2F-4 DNA binding and *trans*-activation. (A) E2F-4/DP-1 heterodimerization *in vivo*. U2OS cells were transfected with expression plasmids (10 μ g each), as indicated above each lane. (Lane 1) Untransfected cells. Cell extracts were prepared as described in Materials and methods and tested for binding to a radiolabeled oligonucleotide containing an E2F site in the presence (+) or absence of the anti-HA mAb 12CA5. (B) DP-1 enhances E2F-4-dependent *trans*-activation. C33A cells (RB^{-/-}) were transfected with 5 μ g of pE2wtCAT, 2 μ g of pCMV β GAL, 200 ng of pcDNA1-DP-1, and the indicated amounts of pCMV-E2F-4. A CAT assay was performed on the resulting cell extracts. In general, 30–50% of an extract of a 10-cm dish of cells was present in each reaction mixture. CAT values were normalized to β -galactosidase units. Results are presented as fold induction vs. quantity of E2F-4-encoding DNA transfected.

dition of anti-HA antibody to the released material supershifted a fraction of the activity (Fig. 5A). Thus, transfected E2F-4 can form a complex with both RB and p107,

Figure 5. Association of E2F-4 with p107 and RB results in decreased E2F-4-dependent *trans*-activation. (A) Binding of E2F-4 to p107 and RB. U2OS cells were transfected with pCMV-HA-E2F-4 (10 μg). Immunoprecipitations were carried out with either N9 (an anti-RB polyclonal antibody) or an affinity-purified anti-p107 polyclonal antibody. The precipitates were treated with DOC, as described in Materials and methods, and the released material was tested for its ability to interact with a radiolabeled oligonucleotide containing an E2F site in the presence (+) or absence (-) of anti-HA mAb 12CA5. (B) Down-regulation of E2F-4-dependent *trans*-activation by p107 and RB. U2OS cells were transfected with pE2wtCAT (5 μg), pCMVβ-GAL (2 μg), pCDNAI-DP-1 (200 ng), pCMV-E2F-4 (500 ng), and 100 ng, 500 ng, or 1 μg of either pCMVp107 or pCMVRb DNA. A CAT assay was performed on extracts of these cultures. CAT values were normalized to β-galactosidase units.

and the E2F-4 molecules in these complexes retain DNA-binding activity.

Whereas E2F-4 can *trans*-activate certain promoters containing E2F sites, cotransfection of an E2F-4 and increasing quantities of a p107 expression plasmid, together with an E2-CAT reporter, led to a significant reduction in CAT activity (Fig. 5B). In the same experiment, introduction of increasing quantities of an RB expression plasmid also led to a decrease, albeit less dramatic, in E2F-4-dependent *trans*-activation function. Repressive effects of this type were not detected when p107 and RB mutants, which do not bind E2F, were tested (data not shown). In some experiments, cotransfection of greater quantities of p107 expression plasmid than the maximum amount analyzed in Figure 5B, led to less reporter action than was detected in the absence of E2F-4 cotransfection (data not shown). Therefore, in addition to negatively regulating exogenous E2F-4 *trans*-activation function, p107 can also suppress endogenous E2F activity, in keeping with earlier published results (Schwarz et al. 1993).

Because these experiments were performed in cells that transiently overproduce HA-E2F-4 and overproduction might, in theory, lead to unphysiologic interactions of HA-E2F-4 with one or more pocket proteins, the ability of endogenous E2F-4 to form complexes with p107 and RB was also tested. Specifically, untransfected U937

(RB⁺) and C33A (RB^{-/-}) cells were metabolically labeled and their extracts immunoprecipitated, in parallel, with RB and p107 antibody. After washing the resulting precipitates, they were dissolved, and the solubilized proteins were reimmunoprecipitated with either E2F-4 or E2F-1 antibody. As can be seen in Figure 6A, E2F-4 was present in the p107 immunoprecipitate in both cell lines. In U937 cells, E2F-4 was also detected in the RB immunoprecipitate, although at a much reduced level compared with that detected in the p107 fraction. In the same experiment E2F-1 was present only in the RB immunoprecipitate. In C33A cells, which fail to synthesize functional RB, no RB/E2F-4 interaction could be detected, as expected. Taken together, these results indicate that E2F-4 interacts, under physiological conditions, with p107. It also interacts with RB, although with apparently lower affinity.

Similar to the results observed previously (Fig. 2C), the E2F-4 isolated from the anti-E2F-4 reimmunoprecipitates, migrated as a heterogeneous set of bands (~57–64 kD). Although these results do not indicate the basis for the heterogeneity, various possibilities exist to explain this phenomenon, including the existence of differentially spliced products and heterogeneously modified products of a single mRNA. Interestingly, in C33A cells, the distribution of bands isolated by direct E2F-4 immunoprecipitation and those that were isolated because

Figure 6. In vivo association of E2F-4 and p107. (A) E2F-4 interacts with p107 in preference to RB in vivo. Extracts of metabolically labeled C33A and U937 cells were immunoprecipitated with a mixture of the anti-p107 mAbs SD9 + SD15, the anti-RB mAb XZ77, or the anti-E2F-4 mAb GG22. The resulting precipitates were then boiled and reimmunoprecipitated by overnight incubation at 4°C, with either GG22 or, where indicated, with the anti-E2F-1 mAb SQ41. (B) E2F-4 is a phosphoprotein. Extracts of [³⁵S]methionine metabolically labeled C33A cells were immunoprecipitated using either monoclonal antibodies against E2F-4 (GG22) or against p107 (SD9 + SD15) and reprecipitated with GG22. Immunoprecipitates were treated as indicated with λ phosphatase with or without the phosphatase inhibitors, vanadate and NaF, or with the inhibitors alone. (C) p107-associated E2F-4 serves as a substrate for p107-associated kinase(s). Extracts of U2OS cells transfected with pCMV-HA-E2F-4 (10 μg) and untransfected U937 cells were immunoprecipitated with a mixture of the anti-p107 mAbs SD9 + SD15. An in vitro kinase reaction was performed on the pellets, as described in Materials and methods. Samples were boiled and reimmunoprecipitated with the same anti-p107 monoclonal mix, anti-E2F-4 rabbit polyclonal antibody, R69 (E2F-4), its preimmune serum (pre), anti-E2F-1 monoclonal (SQ41), or the anti-HA mAb 12CA5 (HA).

they were bound to p107, were not identical. This may mean that p107 selects a specific subset of the existing E2F-4 species. Alternatively, the heterogeneity may be a product of differential phosphorylation, and E2F-4 may be phosphorylated differently when bound to p107 than otherwise. To test the possibility that the heterogeneity was attributable to phosphorylation, we treated E2F-4 immunoprecipitates from metabolically labeled cells with λ phosphatase (NEB), which catalyzes the dephosphorylation of serine, threonine, and tyrosine residues. As seen in Fig. 6B, phosphatase treatment resulted in the appearance of a single, more intense band that corresponded to the fastest migrating E2F-4 species. The effect of the phosphatase was significantly reduced in the presence of phosphatase inhibitors (Fig. 6B). These results suggest that the appearance of multiple E2F-4 bands is attributable to differential phosphorylation.

Because p107-E2F complexes are closely associated with a protein kinase activity (Faha et al. 1992; Lees et al. 1992), we asked whether E2F-4 can serve as a sub-

strate for the p107-associated kinase. To this end, untransfected cell extracts were immunoprecipitated with p107 monoclonal antibody, and the precipitates were allowed to carry out an in vitro kinase reaction in the presence of radioactive [γ -³²P]ATP. Anti-E2F-4 reimmunoprecipitation of the solubilized reaction products was then performed. The E2F-4 precipitates again contained four labeled bands of ~57–64 kD, of which the three more slowly migrating bands were more intense. These bands comigrated with the E2F-4 set detected previously in the p107 precipitate (Fig. 6C). No such bands were detected when an irrelevant antibody was used in the first immunoprecipitation (data not shown). To verify that these bands are E2F-4 species, U2OS cells were transfected with HA-E2F-4 and subjected to the same analysis, except that the reprecipitation was performed with anti-HA antibody. This supernatant reprecipitate also yielded a set of four bands that were, in this case, of slightly slower mobility than those detected in untransfected cells (Fig. 6C), most probably because of the pres-

ence of the HA tag. These results are consistent with the data presented above, suggesting that all of the anti-E2F-4 reimmunoprecipitated bands isolated from the untransfected U937 cell extract were E2F-4 species differing from one another in their state of phosphorylation. Furthermore, these results suggest that E2F-4 can serve as a substrate for the p107-associated kinase, in keeping with the speculation noted above. Although the experiments described in Figure 6C were performed with an E2F-4 polyclonal antibody, identical results were obtained with monoclonal E2F-4 antibody, GG22 (data not shown).

E2F-4 has transforming activity that can be suppressed by binding of a pocket protein

We next examined whether E2F-4 has transforming activity. We decided to analyze, in addition to wild-type E2F-4, an E2F-4 mutant that was refractory to p107 suppression of its *trans*-activation function. To this end, 4 residues (407–411) of the 18-amino-acid pocket protein-binding unit of E2F-4 were deleted. The resulting mutant (E2F-4dl4) could still *trans*-activate but was significantly less repressible by p107 than wild-type E2F-4 (Fig. 7A). Recombinant retroviruses encoding wild-type E2F-4 and E2F-4dl4 were used to infect NIH-3T3 cells. Stable infectants were selected as pools of neomycin-resistant cells that were then analyzed for anchorage-independent growth using a soft agar growth assay. As shown in Figure 7B, E2F-4dl4-infected cultures regularly formed colonies in soft agar, whereas E2F-4-infected cultures did not. Furthermore, the E2F-4dl4-infected cells formed large tumors in nude mice after 2 weeks, whereas at the same time, an identical number of the wild-type E2F-4-infected cells had generated a barely detectable nodule at the site of injection (data not shown). These data imply that, like E2F-1, E2F-2, and E2F-3, E2F-4, also, has transforming activity (G. Xu, D.M. Livingston, and W. Krek, in prep.). They further imply that this activity is normally down-modulated or suppressed by pocket protein binding.

E2F-4 chromosomal localization

The chromosomal location of the E2F-4 gene was determined using fluorescent in situ hybridization with a 15-kb E2F-4 genomic probe. The genomic clone was initially isolated using a fragment of the E2F-4 cDNA to probe a human placenta genomic library (Clontech). A partial sequence of this clone was determined to confirm that it was a segment of the E2F-4 gene (data not shown). Hybridization of this probe to human metaphase chromosomes is illustrated in Figure 8A, and an idiogram summarizing the data for 20 cells is presented in Figure 8B. A signal was consistently observed on both sister chromatids of the long arm of chromosome 16. Analysis of DAPI-banded chromosomes revealed that most signals localized to 16q22, or, in a few cases, to the interface of 16q21 and q22. Chromosome staining was reversed from color to black and white, to increase contrast and to

Figure 7. An E2F-4 derivative confers growth in soft agar. (A) E2F-4dl4 is less responsive to p107 repression than wild-type E2F-4. U2OS cells were transfected with 5 μ g of E2-CAT, 2 μ g of pCMV β GAL, 200 ng of pcDNAI-DP-1, 500 ng of either pCMV-E2F-4 or pCMV-E2F-4dl4, and 10, 50, or 100 ng of pCMVp107. The resulting cell extracts were analyzed by CAT assay. CAT values were normalized to β -galactosidase units. (B) Soft-agar growth of NIH-3T3 cells producing E2F-4 and E2F-4dl4. NIH-3T3 cells were infected with recombinant retroviruses encoding either wild-type or dl4 mutant E2F-4. Resulting infected cell populations, isolated as G418-resistant cell pools, were isolated and characterized as described in Materials and methods. Cell pools were assayed in soft agar growth assays (G. Xu, D.M. Livingston, and W. Krek, in prep.) and photographed after 14 days.

display DAPI bright bands as dark G bands. In the vast majority of chromosome spreads, this site on chromosome 16 was clearly the only site specifically labeled. This is consistent with there being a single copy of the E2F-4 gene in the human genome, mapping to 16q22.

Discussion

A new member of the E2F family, E2F-4, was cloned and found to have structural and functional properties that both liken it to existing members of the family and reveal its unique nature. Like the previously described E2F species, E2F-4 has traditional E2F DNA sequence recognition properties, and its amino acid sequence includes a

Figure 8. E2F-4 chromosomal location. (A) Localization of the E2F-4 gene to human chromosome 16q22 by fluorescent in situ hybridization. DAPI-banded chromosomes were reversed to display DAPI bright bands as dark G bands, with hybridization signals visualized as white spots (arrowheads) present on both chromatids. (B) Idiogram of chromosome 16 depicting the distribution of hybridization signals from the 15-kb genomic E2F-4 phage clone following in situ hybridization analysis of 20 representative metaphases. Each bar denotes the placement of signal on an individual chromosome and is intended to convey the range of precision with which that signal could be placed. The most likely consensus from this analysis placed the gene in the upper region of 16q22, corresponding to 16q22.1, with some signals localized to the interface of bands 16q21 and 16q22.1.

discrete DNA-binding domain in its amino-terminal half. It also carries a potent carboxy-terminal acidic *trans*-activating unit, within which is embedded a pocket protein-binding motif. Like the other three known E2F species, it too forms heterodimeric complexes with a member of the DP family, and complex formation enhances its DNA-binding and *trans*activating functions. Whether heterodimer formation depends on the integrity of its putative zipper-like structure, as is the case for E2F-1 (Krek et al. 1994), remains to be determined.

With regard to its primary structure, its DNA-binding domain, zipper-like structure, carboxy-terminal acidic *trans*-activator, and pocket protein-binding motif are also family signatures. Yet, in vivo, E2F-4 interacts only weakly with RB, unlike E2F-1, E2F-2, and E2F-3 (Helin et al. 1992; Kaelin et al. 1992; Ivey-Hoyle et al. 1993; Lees et al. 1993). This implies that its pocket protein-binding motif, while significantly homologous to that of the other E2F species, may have subtle features that direct it to p107. Alternatively, other structural characteristics of E2F-4 may, along with the pocket protein-binding sequence, contribute to the unique pocket-binding specificity of this protein. Furthermore, E2F-4 lacks an amino-terminal cyclin-binding motif along with a basic region, which the three other E2F species have. Because cyclin A binding to E2F-1, the best understood case, is linked to the negative regulation of its transcriptional activation function in S and G₂ (Krek et al. 1994), it is possible that E2F-4 is not subject to this form of periodic control of its core function. This would be consistent with the fact that E2F-4 gene expression and the prevalence of E2F-4 protein are reasonably constant through the cell cycle, unlike the expression of the E2F-1 gene, which is late G₁ and S phase specific (Kaelin et al. 1992; Krek et al. 1994). Among other possibilities, it may be that E2F-4 function is evenly manifest through the cycle, and if so, some of its target genes might include those whose functions are continuously needed during more than one phase of the

cycle for smooth growth progression and/or for passage through serial cell cycles. Alternatively, E2F-4 might be subject to regulation by cyclin A-kinase, not by direct cyclin A-kinase binding, as is the case with E2F-1 (Krek et al. 1994), but via p107 that interacts independently with both E2F-4 and cyclin A. The observation that E2F-4 is overtly phosphorylated and serves as a substrate for the p107-associated kinase supports this possibility. However, if such cell-cycle phosphorylations occur, they do not result in overt mobility shifts of E2F-4.

The oligoserine array of E2F-4 is also a unique characteristic of the protein. Thus far, we have been unable to link its existence to an aspect of E2F-4 function or regulation of function. Furthermore, in a brief search, there was no evidence of oligoserine amplification in a limited number of human colon tumors marked by CA repeat amplification. This does not eliminate the possibility that such an association exists and, if so, that amplification of the number of serines in the E2F-4 array, in some way, contributes to the evolution of a neoplastic phenotype in certain human tumors. More screening will be needed before such an association can be established or ruled out.

That E2F-4 may be the product of a proto-oncogene is supported by the observation that a derivative can transform at least one line of rodent cells. The existence of this activity is consistent with the recent observation that the other three members of the family are also capable of inducing anchorage-independent growth and monolayer overgrowth of untransformed immortalized cell lines (G. Xu, D.M. Livingston, and W. Krek, in prep.). What is more revealing is the fact that the wild-type protein lacked overt transforming activity in the assay employed, whereas a mutant derivative, less responsive to repression by its pocket protein-binding partner(s), for example, p107, was clearly active in this regard. These data strongly suggest that pocket protein binding to E2F-4 can suppress its transforming function. Better still, because pocket protein binding also led to marked inhi-

bition of E2F-4 *trans*-activating function, it may be that transformation by E2F-4 is, at least in part, a property of its transcriptional activation function. If this is so, transformation suppression by p107 and/or another pocket protein partner, for example, p130, might, in part, be a product of its ability to suppress E2F-4 *trans*-activating function.

It should also be noted that the NIH-3T3 cells used in these transformation assays produce abundant p107. Conceivably, other immortal, but otherwise untransformed cell lines contain lower levels of this E2F-4 regulator. In such cells, it would not be surprising to find that wild-type E2F-4 displays transforming activity, which may, in this case, arise because of a relative deficiency in p107.

Heretofore, little has been discovered that speaks to a unique aspect of p107 function. There is evidence that unique features exist: the distinct timing of its interaction with E2F (Shirodkar et al. 1992), its cell cycle-dependent synthesis (Cobrinik et al. 1993), and differences in the mechanism by which it induces G₁ arrest in RB^{-/-} cells (Zhu et al. 1993). Results presented here reveal a clear biochemical difference between its function and that of RB. That it normally interacts with E2F-4 much more efficiently than RB suggests that RB and p107 transmit different transcriptional signals, perhaps to different genes, via analogous molecular pathways. When a series of E2F-driven promoters were cotransfected with E2F-1 and E2F-4, in parallel, there were significant, albeit not absolute, differences in the responses of some of them to these two related transcription factors (D. Ginsberg and D.M. Livingston, unpubl.). Results of this kind are consistent with the notion that both the transcription factors, themselves, and the pocket proteins that regulate them operate on different genes by analogous, but not identical, biochemical pathways. This notwithstanding, it should also be noted that the transcription assays employed to date are not completely incisive, and the repertoire of E2F-dependent promoters is limited. Therefore, it remains possible that there are certain promoters in which E2F-4 and another E2F family member both have an important role to play, along with their pocket protein partners. If so, there may be subtle timing and/or tissue-specific expression functions, not heretofore apparent, that are the responsibility of these regulator/factor complexes.

Materials and methods

Cell culture and synchronization

Unless otherwise stated, all cells were grown at 37°C in a 10% CO₂-containing atmosphere. HeLa, 293, C33A, U2OS, and the retroviral packaging cell line, Ψ2, were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS, Hyclone). NIH-3T3 cells were maintained in DMEM supplemented with 5% bovine calf serum (BCS, Hyclone). U937 cells were grown at 5% CO₂ in (RPMI) supplemented with 10% FCS. NIH-3T3 cells were growth arrested by incubating cells for 65 hr in DMEM containing 0.5% BCS. Cells were stimulated by addition of BCS to a final concentration of 20% and harvested at appropriate times thereafter.

Plasmids

GST-E2F-4(11) and GST-E2F-4(16) were constructed by one-step PCR using oligonucleotides 636 and 637 (see below) and then subcloning in GST-TK. GST-E2F-4(13-150) was constructed by one-step PCR using oligonucleotides 610 and 560 (see below) and then subcloning in GST-TK. Mammalian expression vectors pCMV-E2F-4(11) and pCMV-E2F-4(16) were generated by replacing the wild-type E2F-1 cDNA in pcDNA1(E2F-1) (Krek et al. 1993) with the 1.2-kb *Bam*HI-*Eco*RI fragment from the respective recombinant GST-E2F-4 plasmids. Similarly, mammalian expression vectors, pCMV-E2F-4(13) and pCMV-E2F-4dl4, were constructed by one-step PCR using oligonucleotides 636, 637, and 636, 936, respectively. The sequences of PCR products were verified by DNA sequencing. HA-tagged E2F-4 and E2F-4dl4 expression vectors were generated by replacing the wild-type E2F-1 cDNA in pcDNA1(HA-E2F-1) (Krek et al. 1993) with the 1.2-kb *Bam*HI-*Eco*RI fragment from the respective pCMV-E2F-4 plasmids. Sequences of the primers were as follows: 636; 5'-GGTGGATCCGCGATGCGGAGGCCGGG-3', 637; 5'-GGGGAATTCTCAGAGGTGAGAACAGG-3', 610; 5'-GGTGGATCCACCATGGGCAC-TCCAAGCCGGCAGCA-3', 560; 5'-GGGGAATTCTCAAGCAAAGCATCTGCAG-3', 936; and 5'-GGGGAATTCTCAGAGGTTGAGAACAGGCACACAGACACCTTCACTCTC-3'. Retroviral vectors carrying E2F-4 and E2F-4dl4 were generated by cloning cDNA fragments encoding HA-tagged E2F-4 and E2F-4dl4 into the retroviral vector pCMV-neo (Bengal et al. 1992). These cDNA fragments were generated by digesting the corresponding pCMV-HA-E2F-4 plasmids with *Hind*III-*Eco*RI. The resulting fragments were isolated, treated with Klenow polymerase to generate blunt ends, and ligated into the *Hpa*I site of pCMV-neo.

Other plasmids used here have been described previously: pCMV-Rb (Qin et al. 1992), pCMVp107 (Zhu et al. 1993), pcDNA1-DP-1 and pcDNA1-HA-DP-1 (Krek et al. 1993), pE2wtCAT (wtE2), pE2(-64/-60, -45/-36)CAT and pE2(-80/-70)CAT (Helin et al. 1992).

Isolation of E2F-4 cDNA

RNA from human T cells was prepared as described previously (Chomczynski et al. 1987). Selection for poly(A)⁺ mRNA was performed using a Pharmacia mRNA purification kit according to the manufacturer's instructions. This poly(A)⁺ mRNA was subjected to a reverse transcription reaction and PCR using the oligonucleotides 5'-GGTGCAGAAGCGGCGCATCTATGAC-3' and 5'-CGCGGATCCATGGAAATCCAGGGGGGTGAGGTCCCCA-3'. Following subcloning into BlueScript and partial sequencing, the PCR product corresponding to E2F-4 was used to screen Nalm6 Zap cDNA library (a gift of A. Bernards, Massachusetts General Hospital, Charlestown) and HeLa λ Zap cDNA library (Xiao et al. 1991) according to standard procedures (Ausubel et al. 1987).

Sequencing

The E2F-4 cDNA sequence was established by sequencing overlapping cDNA inserts using the dideoxy-chain termination method with the Sequenase kit 2.0 (U.S. Biochemical Corp.). Verification of sequences of PCR products was performed using an Applied Biosystems automated sequencer.

Analysis of the CAG repeat region

Extracts containing genomic DNA was prepared from 2×10⁶ cells or 50 μl of whole blood by washing cells three times in TE

buffer and then incubating them for 1 hr at 56°C in 100 ml of K buffer (50 mM KCl, 10 mM Tris-HCl, 2.5 mM MgCl₂, 0.5% Tween-20, 100 mg/ml of proteinase K). Following heating for 15 min at 90°C, 5 µl was used for a PCR with the oligonucleotides 5'-CGGGATCCTGGACACCCGGCCACTGCAGTCTTCTGCC-3' and 5'-CGGAATTCCTCAAAGGAGGTAGAAAGGGT-TGGGTCCG-3' corresponding to nucleotides 879–909 and 990–1017 in E2F-4, respectively. Prior to PCR, one of the oligonucleotides was end-labeled using T4 kinase and [γ -³²P]ATP. PCR products were resolved in a sequencing gel.

FACS analysis

Flow cytometry analysis was performed as described previously (Ewen et al. 1993).

Northern blot analysis

Total RNA was isolated from cells cultured in 15-cm dishes essentially as described previously (Chomczynski et al. 1987). This material was enriched for poly(A) RNA by one round of chromatography on oligo(dT)–cellulose (Pharmacia, mRNA purification kit) according to the manufacturer's instructions. RNA, resolved by electrophoresis in a 1.1% formaldehyde–1.0% agarose gel, was transferred to Hybond N (Amersham) according to the manufacturer's instructions and hybridized to cDNA probes radiolabeled by random priming (Boehringer Mannheim, random-primed DNA labeling kit) as instructed by the manufacturer. For high stringency conditions, filters were prehybridized for at least 4 hr at 42°C with 50% formamide, 5× SSC, 5× Denhardt's solution, 50 µg/ml of denatured salmon sperm DNA, and 25 mM KPO₄ (pH 7.4) and then hybridized to labeled probe at 42°C overnight in the same solution containing 10% dextran sulfate. Filters were rinsed briefly in 2× SSC followed by 4×20-min washes; two washes in 2× SSC, 0.1% SDS at room temperature, then a wash in 1× SSC, 0.1% SDS at 55°C, and finally a wash in 0.2× SSC, 0.1% SDS at 55°C. Low stringency conditions, used for cross-species reactions, were similar except that for prehybridization and hybridization, the formamide content was reduced to 20% and the final two washes were once at 35°C in 2× SSC, 0.1% SDS and once in 1× SSC, 0.1% SDS. Autoradiography was performed at –70°C with intensifying screens. Consecutive hybridizations were performed on filters that had been stripped by boiling in 0.1% SDS for 20–40 min. Efficient removal of labeled probe was checked by autoradiography prior to reprobing.

cDNA probes

The following DNA probes were used in the hybridization reactions noted in this report: Human E2F-4, a 200-bp *Sma*I–*Sfi*I fragment (nucleotides 271–472) of the human cDNA; murine E2F-1, a 1.7-kb partial cDNA corresponding to murine E2F-1 (Li et al. 1994); murine DP-1, a 2.2-kb *Eco*RI–*Xho*I fragment of the murine cDNA (Girling et al. 1993); 36B4, a 0.8-kb *Pst*I fragment of human 36B4 acidic ribosomal phosphoprotein PO (Laborda 1991).

Immunoblotting

Samples of whole-cell extracts, prepared as described previously (Shirodkar et al. 1992), were subjected to electrophoresis (200 µg of cellular protein per lane) in 8% SDS–polyacrylamide gels. After transfer to Hybond-C extra (Amersham), the membranes were blocked for 4–6 hr with 5% nonfat milk in Tris-buffered saline at pH 8.0 (TBS), washed, and incubated overnight at 4°C

with a 1:10 dilution of anti-E2F-4 mAb GG22 hybridoma tissue culture supernatant in TBS plus 3% BSA. Immune complexes were detected as described previously (Ludlow et al. 1989) with alkaline phosphatase-conjugated rabbit anti-mouse secondary antibody (Boehringer-Mannheim).

Bandshift assay

The expression of GST fusion proteins in *Escherichia coli*, their purification on glutathione–Sepharose, and their elution were as described previously (Kaelin et al. 1991). The procedure for the preparation of whole-cell extracts has been described (Krek et al. 1993). E2F DNA-binding assays using an oligonucleotide containing an E2F site derived from the DHFR promoter and release of proteins from immune complexes by DOC treatment followed by E2F bandshift assay were carried out essentially as described (Shirodkar et al. 1992). For antibody perturbation experiments, 1 µl of mAb 12CA5 was added to the reaction mix. Approximately 50 ng of GST fusion proteins were added to the DNA-binding reaction, where indicated.

Transient transfections

Cells were transfected by a modified calcium-phosphate protocol (Qin et al. 1992) with 10 µg of plasmid DNA per 10-cm dish. Cells were harvested 40 hr after transfection and lysed by freeze–thaw in 100 µl of 250 mM Tris-HCl (pH 8.0). Extracts were assayed for β -galactosidase and CAT activities by standard procedures (Gorman et al. 1982; Shirodkar et al. 1992).

Production of antibodies

Polyclonal antibody against gel-purified full-length GST–E2F-4 fusion protein was raised in New Zealand white rabbits by standard techniques (Harlow and Lane 1988). To generate monoclonal antibody, a full-length GST–E2F-4 fusion protein was injected intraperitoneally into BALB/c mice. Spleen cells of positive animals were fused to NS-I cells according to standard procedures (Harlow and Lane 1988). Supernatants from the resulting hybridomas colonies were screened for their ability to immunoprecipitate ³⁵S-labeled, in vitro-translated full-length E2F-4.

Cell labeling and immunoprecipitation

Cells were labeled for 4 hr using 2–3 mCi of [³⁵S]methionine per 10-cm dish of adherent cells, or 1 mCi/ml containing 10⁷ cells for suspension cells, in DMEM containing 10% dialyzed FCS. Cells were lysed for 20 min on ice in 1 ml of TNN buffer (50 mM Tris-HCl at pH 7.4, 120 mM NaCl, 5 mM EDTA, 0.5% NP-40, 50 mM NaF, 0.2 mM sodium orthovanadate, 1 mM DTT, 1 mM PMSF, and 20 µg/ml of aprotinin). The lysate was then centrifuged for 10 min at 10,000g. Following preclearing for 30 min with 60 µl of 50% (vol/vol) suspension of protein A–Sepharose (Pharmacia), supernatants were incubated on a rocker for 1 hr with 100 µl of the indicated antibodies (for mouse monoclonal antibodies the immunoprecipitation was treated with rabbit anti-mouse IgG antibody (Cappel) for 30 min). Fifty microliters of protein A–Sepharose was then added, and incubation was continued for 30 min. Immune complexes were collected by centrifugation and washed four times with TNN buffer. For reprecipitations, washed immunobeads were boiled for 10 min in 100 µl of 50 mM Tris-HCl (pH 7.5), 1% SDS, and 5 mM DTT, followed by addition of 1.3 ml of TNN buffer. Subsequently, samples were incubated with 100 µl of indicated antibodies overnight at 4°C, and immune complexes were collected and

treated as described above. Samples were resuspended in SDS sample buffer, boiled, separated by SDS-PAGE, and detected by autoradiography.

Phosphatase treatment

Immunoprecipitates prepared as described above were washed once and resuspended in 60 μ l of phosphatase buffer, and 500 units of λ phosphatase (NEB) was added to the appropriate tubes and incubated at 30°C for 1 hr. Where indicated, the phosphatase inhibitors vanadate (10 mM) and NaF (5 mM) were included as controls.

In vitro kinase assay

After immunoprecipitation and washing, the beads were washed once with kinase buffer (50 mM HEPES at pH 7.0, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT) and incubated with 30 μ l of kinase buffer containing 10 μ Ci [γ - ^{32}P]ATP (NEN) for 20 min at 37°C. The reaction was terminated by addition of 30 μ l of Laemmli buffer, boiled, diluted, reimmunoprecipitated for 1–2 hr as described above and loaded on a gel.

Genomic DNA cloning

A human placental genomic library (Clontech Laboratories Inc.) was screened with ^{32}P -labeled E2F-4 cDNA by a standard procedure to isolate human E2F-4 genomic DNA fragments. The E2F-4 probe was a product of PCR, using oligonucleotides 5'-GGGATGGGCACCTCCAGG-3' and 5'-CGTGGCGGCGCGCGCC-3', which correspond, respectively, to nucleotides 492–509 and –54 to –38 of E2F-4, as primers and E2F-4 cDNA as template.

Gene localization by in situ hybridization

The fluorescence hybridization procedure for detection of single-locus sequences was used as described previously (Lawrence et al. 1990; Johnson et al. 1991). A 15-kb genomic probe for the E2F-4 gene was labeled by nick translation with digoxigenin-dUTP and detected with rhodamine-conjugated anti-digoxigenin. Hybridization was done overnight at 37°C in 50% formamide, 2 $\times$ SSC to cytogenetic preparations of normal peripheral blood lymphocytes. Metaphase chromosomes were banded for identification by immersion in DAPI. The red fluorescent signal was captured in precise registration with the blue DAPI bands using a triple-band pass filter (Chroma Inc.) and a filter wheel. Twenty metaphase figures were analyzed by two individuals. To facilitate the analysis, images were displayed in black and white to increase contrast, such that the DAPI bands appear as dark "G-bands", and the hybridization signals appear as white spots.

Retrovirus packaging, retroviral infection, and soft agar assays

Ψ 2 cells were transfected with the relevant plasmid using LipofectAMINE reagent (GIBCO BRL) as described (G. Xu, D.M. Livingston, and W. Krek, in prep.). For infection of NIH-3T3 cells, 3 ml of retroviral supernatant, supplemented with 8 mg/ml of polybrene (Sigma), was added to exponentially growing cells and incubated for 4 hr at 37°C. Then, 7 ml of DMEM, containing 5% BCS was added, and incubation was continued for 24 hr. Subsequently, the medium was changed and replaced with selection medium containing 400 mg/ml of neomycin (Sigma). After 10 days of selection, colonies from each plate

were pooled and used for soft agar assay. Both for E2F-4 and E2F4d14, two independent pools of virus-infected cells were obtained and used in this assay. Soft agar assays were carried out as described (G. Xu, D.M. Livingston, and W. Krek, in prep.). Cells in soft agar were photographed after incubation for 14 days.

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