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E2F-4 and E2F-5, two members of the E2F family, are expressed in the early phases of the cell cycle

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ABSTRACT **The E2F transcription factors play a role in regulating the expression of genes required for cell proliferation. Their activity appears to be regulated by association with the retinoblastoma protein (pRb) and the pRb-related proteins p107 and p130. *In vivo*, pRb is found in complex with a subset of E2F components—namely, E2F-1, E2F-2, and E2F-3. Here we describe the characterization of cDNAs encoding two unusual E2Fs, E2F-4 and E2F-5, each identified by the ability of their gene product to interact with p130 in a yeast two-hybrid system. E2F-4 and -5 share common sequences with E2F-1, E2F-2, and E2F-3 and, like these other E2Fs, the ability to heterodimerize with DP-1, thereby acquiring the ability to bind an E2F DNA recognition sequence with high affinity. However, in contrast to E2F-1, E2F-4 and E2F-5 fail to bind pRb in a two-hybrid assay. Moreover, they show a unique pattern of expression in synchronized human keratinocytes: E2F-4 and E2F-5 mRNA expression is maximal in mid-G_1 phase before E2F-1 expression is detectable. These findings suggest that E2F-4 and E2F-5 may contribute to the regulation of early G_1 events including the G_0/G_1 transition.**

E2F/DP heterodimeric transcription factors are likely to be required for regulation of a large number of genes involved in cell proliferation (1, 2). An E2F consensus binding site has been demonstrated to be critical for the control of promoters activated at various different points in the cell cycle including the promoters of the c-*myc* (3, 4), DHFR (5), and *cdc2* (6) genes. This wide spectrum of action may reflect the activities of several distinct E2F heterodimers, whose expression and function are regulated differentially, following distinct, cell cycle-specific schedules.

A number of observations support this model. Cellular E2F activity is associated with several different protein species. Three distinct genes coding for E2Fs (7–11) and three for DPs (refs. 1 and 12; C. L. Wu and E. Harlow, personal communication) have already been identified. Moreover, the expression of the various E2Fs has been reported to be cell cycle dependent. For example, E2F-1 is expressed in the late G_1 phase of the cell cycle (8, 13), clearly later than the induction of some E2F-responsive genes such as c-*myc* (3, 4). Finally, E2F activity appears to be directly and tightly regulated at several successive levels by the cell cycle machinery (14). For example, the E2F-1/DP-1 heterodimer appears to be inactivated through its binding to hypophosphorylated pRb in G_1 (15, 16). Subsequently, phosphorylation of both pRb and E2F-1 (17) in late G_1 results in the release of active E2F-1/DP-1 transcription factors and in the transient expression of E2F-1-dependent genes. During the S and G_2 phases that follow, the direct phosphorylation of E2F-1/DP-1 by cdk2/cyclin A may then cause inactivation (14, 18).

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These processes may well explain the regulation of the three E2F subtypes (E2F-1, -2, and -3) that associate with pRb (11), but they do not address yet other aspects of E2F behavior. Thus, two pRb-related proteins, p107 (19) and p130 (20, 21), also form complexes *in vivo* with cellular E2Fs (1, 22). Moreover, the E2Fs associated with p107 and p130 may be distinct from those bound to pRb (23). In certain cell types, complexes containing E2Fs and p130 are most prominent in G_0 and during much of G_1, in contrast to the behavior of pRb, whose association with E2F factors is barely demonstrable (22, 24). Provoked by this observation, we developed a strategy to isolate p130-associated E2Fs that are expressed in G_0. We report here the cloning of two cDNAs encoding E2F-like transcription factors.§

MATERIALS AND METHODS

Cell Culture. Human WI38 fibroblasts (American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO) with 15% heat-inactivated fetal calf serum (FCS). For synchronization in G_0, confluent cell monolayers were maintained in DMEM/0.1% FCS for 3 days. Human HaCaT keratinocytes were grown as described (25).

Yeast Strain. Mav103 (*MATa ura3-52 leu2-3,112 trp1-901 his3Δ200 ade2-101 gal4Δgal80ΔGAL1:LacZ GAL1:HIS3@lys2 SPAL9:URA3*) will be described elsewhere (M.V., unpublished data).

Plasmids. Details of all constructions are available upon request. The "bait" (pPC97 and pPC97-CYH2) and "prey" (pPC86 and pPCS86) centromeric plasmids (ref. 26; M.V., unpublished data) carry the DNA binding domain (DB) (aa 1–147) and the transactivation domain (TA) of Gal4 (aa 768–881) as well as the *LEU2* and *TRP1* selectable markers, respectively. Fusions to DB were as follows: pPC97-130, aa 427–1139 of p130 (20, 21); pPC97-*CYH2*-Rb, aa 302–928 of pRb (27); pPC97-*CYH2*-RbΔ22, aa 281–894 of pRb pocket mutant Δ22 (28); pPC97-DP-1, aa 1–410 of DP-1 (29). Fusions to TA were as follows: pPCS86-E2F-1, aa 159–437 of E2F-1 (7); pPC86-DP-1, aa 1–410 of DP-1 (29); 1301, aa 1–413 of E2F-4; 1305, aa 22–345 of E2F-5. pKS1-E2F-4 and pSK22-E2F-5 are pBluescript SK+ and KS+ plasmids (Stratagene) containing full-length E2F-4 and E2F-5, respectively.

G_0 cDNA Library in pPCS86. Polyadenylylated mRNAs were prepared from 8×10^8 serum-starved WI38 cells and *Xho* I/oligo(dT)-primed cDNAs were synthesized by a modification of the procedure described by Gyuris *et al.* (30). A *Not* I/*Eco*RI adaptor (Invitrogen) was ligated at the 5′ end of the

Abbreviations: DB, DNA binding domain; TA, transactivation domain; 3-AT, 3-aminotriazole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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§The sequences reported in this paper have been deposited in the GenBank data base [accession nos. U15641 (E2F-4) and U15642 (E2F-5)].

cDNAs. Half of *Not* I/*Xho* I-digested cDNAs were size-selected on Sepharose CL4B spin columns (Pharmacia) and the other half were selected on Sephacryl S500 (Pharmacia). Both eluates were combined and ligated into pPCS86 cut with *Not* I and *Sal* I. Electrocompetent *Escherichia coli* DH10B (BRL) were transformed leading to 3×10^6 individual colonies, >85% of which contain a cDNA insert whose average size is 1.4 kb.

Two-Hybrid Screen. In this work, several technical modifications have been added to previously developed systems (26, 30–32). Details of these modifications will be described elsewhere (M.V., unpublished data). In short, the screen was performed in the yeast strain Mav103. This Gal4-deleted strain is auxotrophic for histidine, uracil, leucine, and tryptophan and carries three chromosomally integrated reporter genes whose expression is regulated by different Gal4 responsive promoters: *GAL1:HIS3, SPAL10:URA3, GAL1:LacZ.* The prey library and pPC97-130 were introduced into Mav103 by the lithium acetate method (30). Transformants were plated onto synthetic complete (SC)-Leu-Trp medium, replica-plated after 2 days onto SC-Leu-Trp containing 10 mM 3-aminotriazole (3-AT; Sigma) to select for *GAL1:HIS3*-dependent His prototrophy (32), and subsequently replica-plated again onto SC-Leu-Trp containing 30 mM 3-AT. Positive clones were picked after an additional 3 days of incubation. Prey plasmids were rescued from yeast by electroporation of *E. coli* XL1 Blue with total yeast DNA (30). Mav103 yeast cells containing pPC97-130 or pPC97-DP1 were retransformed with these prey plasmids and activation of all three reporters was tested as follows: (*i*) His prototrophy on SC-His plus 30 mM 3-AT; (*ii*) β-galactosidase activity, using a filter lift assay (32); (*iii*) *URA3* activation on SC-Ura.

Cloning of Full-Length cDNAs and Sequence Analysis. A human fetal liver 5′ stretch cDNA library in λDr2 (Clontech) was screened for E2F-4 with a 5′ end *Not* I/*Apa* I fragment purified from prey clone 1301 and for E2F-5 with a *Not* I/*Eco*RV fragment purified from clone 1305. All cDNAs were sequenced using Sequenase 2.0 (United States Biochemical). Amino acid sequence comparisons and construction of the phylogenic tree were performed using GAP, PILEUP, DISTANCES, and GROWTREE programs from GCG using default parameters (version 8, Genetics Computer Group, Madison, WI).

***In Vitro* Translation.** Two micrograms of pSK22.E2F-5 and pKS1.E2F-4 and pBS-RBP3 [E2F-1 (7)] were *in vitro* transcribed (T7 polymerase) and translated using a TNT-coupled reticulocyte lysate system (Promega) according to the manufacturer's protocol in a final volume of 50 μl per assay.

Mobility-Shift Assays. The mobility-shift assays were performed as described (22). Unlabeled *in vitro* translated E2F reaction mixtures (1–5 μl) were incubated with gel-purified ^{32}P-end-labeled synthetic double-stranded oligonucleotide probe (0.5 pmol/ml) representing the −82 to −40 sequence of the human *MYC* P2 promoter (22). Competitive binding reaction mixtures included a 20-fold molar excess of unlabeled double-stranded oligonucleotide representing: (*i*) −53 to −72 of the adenovirus E2 promoter (plus strand, 5′-AGCTAGTTTTCGCGCTTAAATTT-3′) and (*ii*) the corresponding point mutant (5′-AGCTGTTTTCGAGCTTAAATTT-3′).

Northern Blot Analysis. Total RNA (15 μg) extracted as described (25) was resolved by formaldehyde/1% agarose gel electrophoresis and transferred onto Hybond N membrane (Amersham). The same membrane was hybridized successively with the following ^{32}P-labeled human probes prepared with PCR or restricted fragments: (*i*) E2F-1, *Not* I/*Xho* I 5′ fragment from pPCS86-E2F-1, (*ii*) E2F-4, *Not* I/*Apa* I 5′ fragment from pPC86-E2F-4, (*iii*) E2F-5, *Eco*RV central fragment from pPC86-E2F-5, (*iv*) DP-1, *Sma* I/*Pst* I central fragment from pPC86-DP-1. The amount of RNA in each lane was normalized by hybridization with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) full-length cDNA. The signals were quantified by densitometric scanning and plotted after normalization.

RESULTS

Cloning of E2F-4 and E2F-5, Two Members of the E2F Family. A yeast two-hybrid screen was used to identify proteins that are expressed in quiescent human fibroblasts and are able to interact with human p130. The assay was based on reconstituting a functional transcriptional activator from two separate fusion proteins (31): (*i*) as bait, the DB of Gal4 was fused to the "large pocket" of p130 (aa 427–1139)—this domain shows extensive similarities with comparable regions in p107 and pRb where it is required for E1A and E2F binding (20, 21, 26); (*ii*) as prey, the TA of Gal4 fused to the products encoded by a cDNA library generated from serum-starved WI38 human fibroblasts (26).

The prey library was introduced into the yeast reporter strain Mav103 containing p130 bait and 5×10^5 transformants were selected for reporter-dependent histidine prototrophy. Prey plasmids were recovered from 19 growing colonies and used to retransform Mav103 containing p130 bait. Nine transformants showed the same histidine prototrophy and scored positive for the two other reporters present in Mav103, showing β-galactosidase activity and prototrophy for uracil. Among these prey cDNAs, 5 were able to induce a similar reporter activating phenotype in Mav103 expressing a full-length human DP-1 bait. These clones, 1301–1305, were likely to encode members of the E2F family of transcription factors and were further characterized.

Sequence analysis confirmed that all these clones encoded unusual E2F-like molecules, having homology to human E2F-1, E2F-2, and E2F-3 (7–11). Four cDNAs were derived from the same gene, termed E2F-4. The fifth one was referred to as E2F-5. A 5′ stretch cDNA library of human fetal liver was further screened with 5′ DNA probes of E2F-4 and E2F-5. The longest clones obtained were sequenced (Fig. 1). E2F-4 cDNAs contained a 1239-bp open reading frame encoding a 413-aa protein (predicted mass, 44 kDa). E2F-5 cDNAs contained a 1035-bp open reading frame encoding a 345-aa protein (predicted mass, 37.5 kDa).

Structure of E2F-4 and E2F-5: Comparison with Other E2Fs. The overall relatedness between E2F-4 and E2F-5 (69% identity, 80% similarity) is stronger than their respective relatedness to the other members of the E2F family (between 36% and 40% identity and 52% and 60% similarity). However, all E2Fs are structurally similar, having numerous conserved sequence blocks (Fig. 1*a*). As previously determined experimentally for E2F-1 and/or E2F-2, some of these blocks correspond to distinct functional domains. Region B in Fig. 1*a* encompasses the DB (7, 10, 11, 29). It shows the highest level of conservation between the five members of the family and was used to construct an evolutionary tree of all known E2F family members [including *Drosophila* E2F (33, 34) and DP-1 (12)]. This diagram (Fig. 1*b*) clearly shows that E2F-4 and E2F-5 constitute a subclass of factors evolutionarily distinct from the other E2F-like molecules.

Regions C and D are reported to be necessary for the heterodimerization of E2F-1 with DP-1 (29). Region C is likely to be involved in this association since it shows a conserved heptad repeat structure with hydrophobic residues at position 7, a motif previously described to mediate protein–protein interaction (35). Domain E is of unknown function and has been cited previously as a larger region called a "marked box" (11). Finally, the C-terminal domain (region F) of E2F-1 is involved in its binding to pRb (7). It is likely that this region is also involved in the binding of E2F-4 to p130, since one of the cDNAs that we isolated in our two-hybrid screen encoded only the last C-terminal 114 aa of E2F-4. By extension, it may

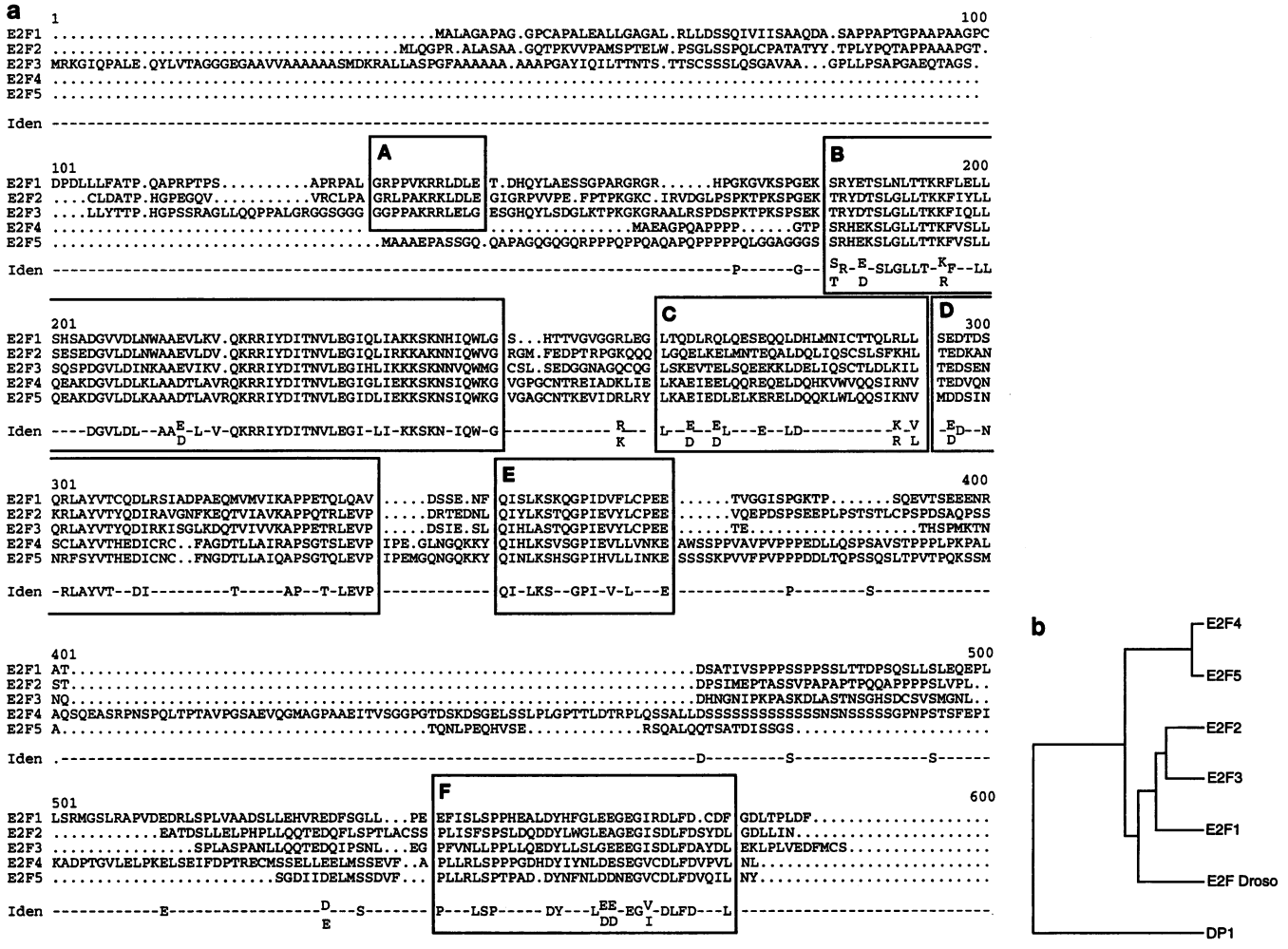

FIG. 1. (*a*) E2F-4 and E2F-5 amino acid sequences. PILEUP comparison with E2F-1, E2F-2, and E2F-3. Iden, residues that are identical in at least four of five proteins. Boxes indicate domains of homology referred to in the text as follows: A, cyclin A binding domain; B, DB; C and D, DP dimerization domain; C, heptad repeat; E, Marked box; F, pocket protein binding domain. (*b*) Evolution tree of the E2F family based on comparison of the DBs of human E2F-1, E2F-2, E2F-3, E2F-4, E2F-5, DP-1, and *Drosophila* E2F.

represent the domain carried by all E2Fs that is required for their binding to the pockets of pRb and related proteins.

E2F-4 and E2F-5 are distinguished from other members of the family in that their N termini are shorter, lacking a domain (Fig. 1*a*, domain A) that in E2F-1 binds cyclin A (14). In addition, E2F-4 has a longer spacer segment between domains E and F containing a trinucleotide repeat (CAG) encoding a stretch of 13 consecutive serines. This spacer, which is serine and acidic residue-rich (33–42%) in all E2Fs, is likely to function as a TA (8–10, 11, 29).

E2F-4 and E2F-5 DNA Binding: Synergistic Effects with DP-1. To determine whether E2F-4 and E2F-5 share DNA binding characteristics with other E2Fs, we tested their respective abilities to bind a consensus E2F DNA binding site and to exhibit cooperative DNA binding with DP-1 as shown previously for E2F-1 (31). Gel mobility-shift assays were performed with nonradioactive *in vitro* translated E2F-1, E2F-4, E2F-5, and DP-1 proteins mixed with a ^{32}P-labeled oligonucleotide probe representing the E2F DNA binding site of the human c-*MYC* P2 promoter (3).

As shown in Fig. 2, E2F-1, E2F-4, and E2F-5 but not DP-1 caused a detectable retardation when preparations of each were mixed with the probe. This shift was strongly enhanced when DP-1 was added to E2F assays. The observed shifts were specific, since they were blocked by competition with an excess of unlabeled wild type but not mutant E2F binding site oligonucleotide. Thus, the binding properties of recombinant E2F-1, E2F-4, and E2F-5 to the consensus E2F DNA binding site appear to be very similar in this *in vitro* assay.

Cell Cycle Expression of E2F mRNAs in Human Keratinocytes. The identification of E2F family members raised the possibility that the various E2Fs perform different functions at distinct times in the cell cycle. To address this possibility, we first compared the timing of their expression during the early phases of the cell cycle. HaCaT human keratinocytes, synchronized in G_0 by serum deprivation, were restimulated with 10% serum. Their entry into S phase was then monitored by measuring incorporation of BrdUrd into DNA (25). As shown in Fig. 3*B*, these cells entered S phase ≈14 hr after serum stimulation. Total RNAs were prepared at 2-hr intervals for Northern blotting analysis. The same blot was hybridized successively with E2F-1, E2F-4, E2F-5, DP-1, cyclin E, and GAPDH cDNA probes (Fig. 3*A*). Autoradiographic signals were quantified and plotted after normalization with GAPDH signals (Fig. 3*B*).

As previously described (8, 13), E2F-1 mRNA was almost undetectable in G_0 and early G_1 and first appeared 7–9 hr after serum stimulation, reaching its maximum level at 15 hr when cells were entering S phase. Interestingly, this expression pattern follows exactly that of cyclin E mRNA (data not shown; see ref. 25). In contrast, the E2F-4 probe detected an abundant 2.9-kb mRNA that was already detectable in G_0. Upon serum stimulation, the E2F-4 mRNA started to increase at 5 hr, reached its 2- to 3-fold higher level in mid-G_1 at 9 hr, and

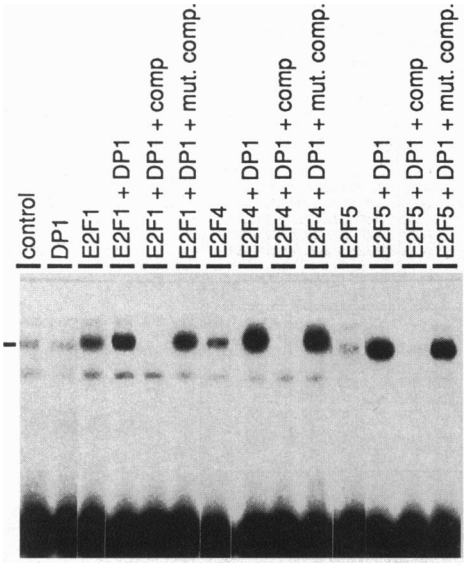

FIG. 2. Effect of DP-1 on DNA binding of E2F-1, E2F-4, and E2F-5. A ^{32}P-labeled oligonucleotide containing a single E2F binding site was incubated with *in vitro* translated DP-1, E2F-1, E2F-4, and E2F-5 alone or with E2F/DP-1 combinations as indicated. Wild type (comp) or mutant (mut. comp.) competitor was added at 20-fold excess with E2F/DP-1 combinations.

declined thereafter, returning to its initial G_0 level as the cells entered S phase. The expression of the E2F-5 2.8-kb mRNA followed similar kinetics. However, its levels of expression in G_0 and in S phase were much lower than those of E2F-4 at the same time points, although its peak of expression was reaching 12-fold the basal level.

Finally, we found that DP-1 mRNA levels increased 3-fold by mid-G_1, showing kinetics that parallel those of E2F-4 and E2F-5. However, after reaching its maximum, DP-1 mRNA did not significantly decrease. Interestingly, the broad peak of DP-1 expression from mid-G_1 to S phase covered the expression peaks of both E2F-4/E2F-5 in mid-G_1 as well as that of E2F-1 in late G_1 and S phase. This supports the notion that DP-1 might represent a common partner for all these E2Fs (1).

E2F Interaction with pRb, p130, and DP-1 in a Two-Hybrid Assay. To pursue the comparison between E2F-4 and -5 and E2F-1, we investigated their abilities to bind to DP-1 and to the pocket proteins pRb and p130 in a yeast two-hybrid interaction assay (Fig. 4). The baits used in these two-hybrid screens, each fused to the DB of Gal4, were as follows: (*i*) The pocket domain of pRb required to bind E1A and E2F *in vivo* (16, 28); (*ii*) the corresponding domain of the pRb mutant Δ22 that fails to bind E1A and E2F *in vivo* (16, 28); (*iii*) the pocket domain of p130 (20, 21); (*iv*) the full-length DP-1. Comparable levels of expression of the various baits were observed by using antibodies directed against Gal4 (data not shown). On the prey side, the properties of the cDNAs 1301 (E2F-4) and 1305 (E2F-5) obtained from the initial two-hybrid screen were compared with those of DP-1 and E2F-1. Each of these was fused to the TA of Gal4. All possible bait/prey combinations were tested in Mav103 for *GAL1:HIS3*-dependent histidine prototrophy (Fig. 4).

As shown previously (32), the reporter-dependent growth observed in this assay is directly correlated with the strength of interaction between bait and prey. The matrix shown in Fig. 4 summarizes the various combinatorial interactions. As observed *in vivo*, E2F-1 interacted strongly with DP-1. It also bound pRb and p130 and failed to interact with the pocket mutant of pRb. In contrast to E2F-1, E2F-4 and E2F-5 both bound strongly to p130 and to DP-1 but not to pRb. In addition, preliminary data show that in the same assay, E2F-4 and E2F-5 also interacted with p107 (data not shown). Finally, DP-1 did not interact either with itself or with the pocket proteins. The specific affinity showed by E2F-4 and E2F-5 for p130 in this assay combined with their early expression in G_1 suggests that *in vivo* these E2F proteins may account for much of the p130-associated E2F activity detected during G_0 and G_1 (22).

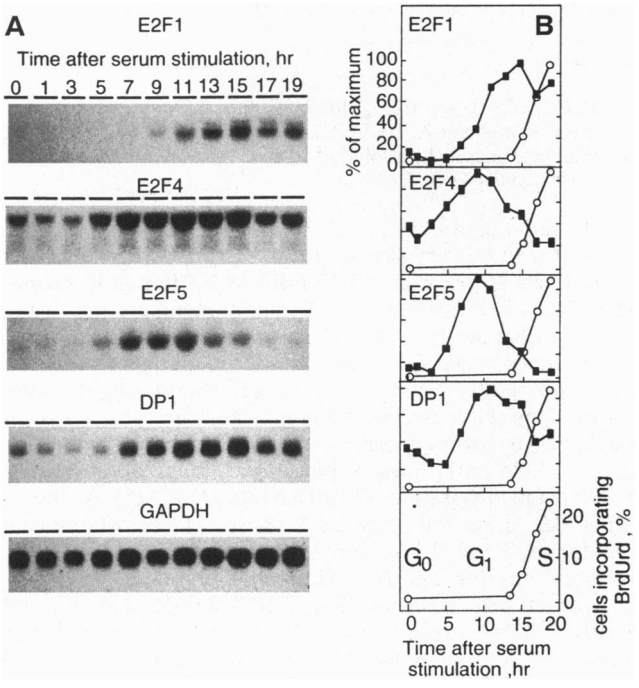

FIG. 3. Cell cycle expression of E2F-1, E2F-4, E2F-5, DP-1, and GAPDH mRNAs in synchronized human keratinocytes. (*A*) Three-day serum-starved HaCat cells were harvested at the indicated times after 10% serum stimulation. Northern blot analysis was performed on total RNAs and probed sequentially with ^{32}P-labeled human cDNAs encoding E2F-1, E2F-4, E2F-5, DP-1, and GAPDH. (*B*) Expression of E2F-1, E2F-4, E2F-5, and DP-1 normalized to GAPDH mRNA by densitometric scanning of the gel in *A* (■). Right axis indicates DNA synthesis monitored in the same experiment by measurement of BrdUrd incorporation in DNA (○).

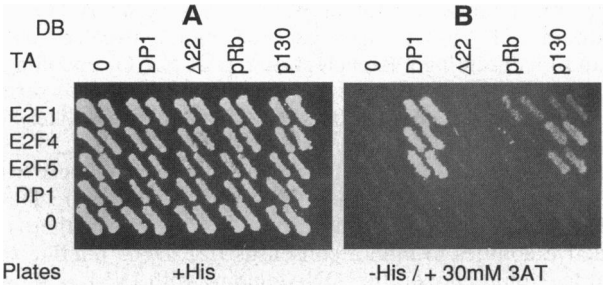

FIG. 4. Interactions of E2F with DP-1, p130, and pRb in a two-hybrid assay. Mav103 yeast strain containing chromosomally integrated reporter genes whose expression is regulated by different Gal4 responsive promoters: *GAL1:HIS3*, *SPAL10:URA3*, *GAL1:LacZ* was cotransformed with all possible combinations of the following baits and prey. Baits (DB) are built in the centromeric expression vector pPC97 by fusion of a Gal4 DB to DP-1, p130, pRb, and pRb pocket mutant Δ22. Prey (TA) are built in pPC86 by fusion of a Gal4 TA to E2F-1, E2F-4, E2F-5, and DP-1. Amino acid positions of these fusions are described in *Materials and Methods*. Reporter activation was tested as follows: Yeast growing on permissive plates (SC-Leu-Trp) (*A*) were selected for *GAL1:HIS3*-dependent His prototrophy by replica plating on SC-Leu-Trp-His plus 30 mM 3-AT plates (*B*).

DISCUSSION

The two E2Fs described here, E2F-4 and E2F-5, exhibit structural properties that clearly link them to the E2F family, including a highly conserved DB, a conserved DP-1 heterodimerization domain, and a potential C-terminal acidic TA within which is embedded a putative pocket protein binding motif. Moreover, like the other E2Fs, E2F-4 and E2F-5 interact with DP-1, forming heterodimers with an enhanced capacity to bind DNA.

E2F-4 and E2F-5 share several distinctive properties that distinguish them from the other E2Fs. Examination of their amino acid sequences reveals that they are more closely related to each other than they are to other members of this family of transcription factors. In addition, the early and abundant expression of E2F-4 and E2F-5 mRNA raises the possibility that the encoded proteins are already present in G_0 and in G_1 when E2F-1 has not yet been synthesized (8, 13). Moreover, they both fail to interact with pRb in our two-hybrid assay, unlike E2F-1 (and E2F-2; unpublished data). Since E2F-4 and E2F-5 do bind p130 and p107 in the same assay, we propose that these E2Fs might be the hitherto unidentified physiological G_1 E2F partners of these pRb-related proteins (22). However, we recognize that these differential interactions observed with the yeast two-hybrid assay do not offer a definitive proof of interaction within the mammalian cell. Further experiments in mammalian cell lines are required to determine whether p130 and/or p107 controls these E2Fs and how these factors contribute to G_1 gene expression.

The recent example of the E2F-1/pRb activation–inactivation cycle strongly suggests that phosphorylation of both partners by cyclin-dependent kinases is involved in the regulation of their association (14, 18). It is tempting to propose that a similar mechanism regulates p130/E2F-4 and/or p130/E2F-5 association in G_1. Two recent observations may support this model. First, *in vivo*, some E2F/p130 complexes contain cyclin E-associated kinase (22); second, p130 associates *in vitro* with cyclins D, E, and A (20). However, it is not known whether these kinases are able to phosphorylate p130 and the associated E2Fs or whether the appearance of these kinases in E2F/p130 complexes triggers the activation of an E2F-dependent transcription. Our working model is that these E2F factors escape p130 control in a cyclin-dependent manner at different points during the G_0 to S progression. The liberated E2F-4/DP and E2F-5/DP may then be involved in transcription of early E2F-dependent genes like the c-*myc* gene (3) as well as that of late G_1 genes required for the G_1/S transition, as cyclin E or even E2F-1 itself (36, 37). The identification of these E2Fs as putative p130 partners will allow us to directly address these questions.

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