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Biochemistry

Transcription factor E2F binds DNA as a heterodimer

(retinoblastoma/pRB/protein)

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ABSTRACT **E2F is a mammalian transcription factor that appears to play an important role in cell cycle control. DNA affinity column-purified E2F from HeLa cells reproducibly exhibits multiple protein bands when analyzed by SDS/PAGE. After electrophoretic purification, electroelution, and refolding of the individual protein components, the E2F DNA binding activity of the individual proteins was poor. However, upon mixing the individual components together, a dramatic (100- to 1000-fold) increase in specific DNA binding activity was observed. The five protein bands isolated can be separated into two groups based on apparent molecular mass. Optimal reconstitution of activity requires one of the two proteins found in the group of larger molecular mass (≈60 kDa) and one of the three proteins in the smaller-sized group (≈50 kDa). The reconstituted heterodimer is identical to authentic affinity-purified E2F by three criteria: DNA-binding specificity, DNA footprinting pattern, and binding to the retinoblastoma gene product. A recently cloned protein with E2F-like activity, RBP3/E2F-1, is related to the protein components of the group of larger molecular mass, as determined by Western blot analysis and reconstitution experiments. These data suggest that E2F, like many other transcription factors, binds DNA as an oligomeric complex composed of at least two distinct proteins.**

E2F is a mammalian transcription factor that was initially detected as a cellular factor involved in activation of the adenovirus E2 promoter (1–4). Subsequent studies have revealed that E2F may also play a critical role in cell cycle regulation of uninfected mammalian cells. The promoter regions of several important cellular genes, including c-*myc*, N-*myc*, c-*myb*, and *DHFR* contain sequences similar to the E2F binding site of the adenovirus E2 promoter (5). For c-*myc* and *DHFR*, this promoter sequence has been shown to be sufficient to activate gene transcription (5, 6).

E2F activity in mammalian cells appears to be regulated by formation of multiprotein complexes with other cellular factors. E2F has been shown to bind to the retinoblastoma growth suppressor gene product (pRB), p107 (a pRB homolog), cyclins, and cdk2. The formation and dissociation of these complexes correlates with progression through the cell cycle and may be regulated by protein phosphorylation (7–13). Recent experimental evidence suggests that the complexed E2F is the functionally inactive form, while free E2F is the transcriptionally active form (14, 15).

At the time the current study was initiated, E2F was defined solely by its activity and functional properties, rather than as a distinct protein. Two of these biochemical properties are (*i*) that E2F binds DNA in a sequence-specific manner (consensus sequence, TTTCGCGC) in a gel-shift assay, and (*ii*) that the mobility of this complex is shifted by pRB and the other proteins cited above. Recently, workers in two different laboratories published the sequence of a clone [designated retinoblastoma-binding protein 3 (RBP3), RBAP1, or E2F1] that displays many of the properties of authentic E2F (16, 17), including sequence-specific DNA-binding activity and binding to pRB. Nevertheless, antibodies to RBP3 protein supershifted only a subset of the E2F protein in a gel-shift assay, suggesting that the cloned protein represents a subset or a component of cellular E2F(s) (17).

Following the purification of E2F for biochemical studies, we reproducibly observed multiple SDS/PAGE protein bands in the DNA affinity column eluate. In the current study, we report that five of these bands may represent components of authentic E2F. Optimal reconstitution of site-specific DNA-binding and pRB-binding activities requires at least two of these gel-purified components. These results suggest that, like many other transcription factors, E2F functions as a heterodimer composed of two protein subunits.

MATERIALS AND METHODS

Purification of E2F. Human E2F was purified from HeLa cells by methods similar to those described (18, 19). Briefly, whole cell lysate was fractionated over heparin-agarose and peak E2F fractions were then applied to a high-performance anion-exchange column (Bio-Rad MA7Q). Detergent {CHAPS; 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate} was added to a final concentration of 0.1%. The E2F-containing fractions were pooled and diluted to give a final KCl concentration of 200 mM. Following concentration using a YM30 membrane (Amicon), the E2F was bound to a sequence-specific DNA affinity column equilibrated in a buffer containing 20 mM Hepes (pH 7.9), 1 mM EGTA, 10% (vol/vol) glycerol, 0.1% CHAPS, 0.02% sodium azide, and 200 mM KCl. After washing with equilibration buffer containing 280 mM KCl, E2F was eluted in buffer containing 400 mM KCl. The eluate was diluted 1:2 before reapplication on the affinity column. After washing with equilibration buffer, E2F was eluted with 20 mM $MgCl_2$. A dominant DNA-binding species was observed in the gel-shift assay using an E2F probe (see *Results*). The E2F–DNA band was eliminated by competition with wild-type but not mutant oligonucleotide and was resistant to treatment with 0.6% deoxycholate, indicating that it represents free E2F.

E2F components were separated on 8% bisacrylamide Laemmli gels (NOVEX, San Diego) and visualized by Coomassie blue staining. Individual bands were separated with a razor blade, electroeluted into 20 mM ammonium bicarbonate/0.01% SDS with an Amicon Centrilutor, and concentrated 10:1 in Centricon 30 concentrators (Amicon).

Expression and Purification of RBP3 Proteins. RBP3-(89–437) and RBP3-(110–191) were expressed as glutathione-*S*-

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Abbreviations: GST, glutathione-*S*-transferase; RBP3, retinoblastoma-binding protein 3.

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transferase (GST) fusion proteins. Plasmids pGEX30X-RBP3(89–437) and pBSK RBP3(1–437) and rabbit polyclonal anti-RBP3-(409–426) IgG were kindly provided by K. Helin (Massachusetts General Hospital, Boston). The DNA coding for aa 110–191 was subsequently subcloned into pGEX2T (Pharmacia). GST fusion proteins were affinity purified on glutathione resin (Pharmacia). For complementation with individual E2F bands, the GST portion was removed with thrombin (Calbiochem). RBP3-(89–437) was subsequently purified on a preparative SDS gel and electroeluted as described above. Full-length RBP3 was prepared by fusing aa 1–437 with a C-terminal tripeptide α-tubulin epitope followed by affinity purification (20).

Gel-Shift Assay and Binding Competitions. E2F was detected by a DNA gel-shift assay (21). E2F and [^{32}P]DNA oligomers were incubated at room temperature for 20 min in 20 mM Hepes, pH 7.9/1 mM $MgCl_2$/0.1 mM EDTA/40 mM KCl/10% glycerol/0.15% Triton X-100/1 mM dithiothreitol (DTT) (buffer A)/5 mg of bovine serum albumin (BSA) per ml. Denatured salmon sperm DNA at 80 μg/ml was included with crude but not with affinity-purified E2F. Protein–DNA complexes were separated on precast 6% gels (NOVEX). The E2F DNA probes were synthesized by Midland Certified Reagent (Midland, TX). They were based on the adenovirus type 5 E2 promoter but contained only one of the two E2F binding sites (5′-TAGTTTTCGATATTAAATTTGA-GAAAGGGCGCGAAACTAG-3′) (E2F site underlined) or a single palindromic site derived from the DHFR promoter (5′-TAGTTTTCGATATTAAATTTGAGTTTTCGCGC-GAAACTAG-3′). Unlabeled competitor DNA contained either the wild-type E2F site or a mutated site (TATCGAAA).

pRB–E2F complexes were reconstructed by using affinity-purified E2F and either a recombinant 60-kDa fragment of pRB (pRB60) (22) or full-length pRB105. Affinity-purified pRB60 contains the viral oncoprotein-binding pocket and has full E2F-binding activity (23). Baculovirus-expressed pRB105 was a gift from Carol Prives (Columbia University) and was affinity purified as described for pRB60 (22). Unless otherwise indicated, pRB was present at a final concentration of 400 ng/ml during the incubation of E2F and DNA.

Renaturation Experiments. Electroeluted protein samples were refolded by diluting them 1:15, either individually or combined, to give the following final concentrations: 5.2 M guanidine hydrochloride, 60 mM Tris·HCl (pH 8.5), 55 mM NaCl, 5 mM DTT, 0.9 mM EDTA, 0.7 mM NH_4HCO_3, 0.001% SDS, and 36 μg of BSA per ml. After 30 min at room temperature 7 vol of cold buffer A with 5 mM DTT was added and incubation was continued for 30 min on ice. After centrifugation at 4°C the supernatants were transferred into Microcon-10 tubes (Amicon) and concentrated ≈20-fold at 4°C. Samples were diluted to the original volume with buffer A and DTT, concentrated a second time, and tested for E2F-binding activity.

RESULTS

E2F protein was purified from HeLa cell lysates. SDS/PAGE analysis of the affinity column eluate reproducibly yielded multiple protein bands, with five distinct bands identified in the 50- to 60-kDa range (Fig. 1*A*). Several additional chromatography steps, including reapplication to the DNA affinity column in the presence of an excess of nonspecific single- or double-stranded DNA or in the presence of 0.1% deoxycholate, did not resolve the proteins (data not shown). These observations suggest that each of the proteins contacts DNA directly.

The affinity-purified E2F resulting from ≈200 liters of HeLa cells was further purified by SDS/PAGE. Initially the lane containing E2F was cut into 12 slices, and the proteins were electroeluted, renatured, and assayed for E2F activity.

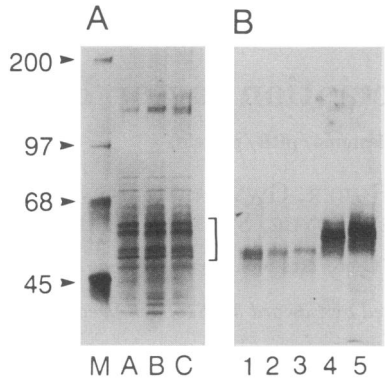

FIG. 1. Silver-stained SDS gels of DNA affinity-purified E2F from HeLa cells. (*A*) Molecular size markers (lane M) and three different batches of E2F (lanes A–C). Region of gel with E2F activity is bracketed. (*B*) Protein bands 1–5 with E2F DNA-binding activity (lanes 1–5) purified by electroelution. Numbers on left are kDa.

Only the proteins in the 50- to 60-kDa range displayed any E2F activity (data not shown), similar to previous reports (18). Five protein bands in that size range (numbered 1 to 5, from smallest to largest) were excised and individually electroeluted from the gel slices (Fig. 1*B*). The eluted proteins were then concentrated, refolded, and assayed for E2F activity in the gel-shift assay. All five proteins bound to the E2F probe, displaying the appropriate selectivity for binding the wild-type, but not the mutant, oligonucleotide (Fig. 2*A*). The protein eluted from each SDS/PAGE band yielded a distinct complex in the gel-shift assay, with the mobility correlating with the apparent molecular mass. The distinct location of each complex confirms that the preparative SDS/PAGE step resolved the proteins in the 50- to 60-kDa molecular mass range into five different active components.

The recovery of E2F activity from the preparative SDS/PAGE and refolding steps was low (0.1–1% for each band). Several additional refolding protocols were tested but did not improve the yield of activity (data not shown). Surprisingly, none of these individually refolded proteins appeared to bind the retinoblastoma gene product (pRB60), as monitored by pRB60-dependent supershifting of the protein–DNA complex in the gel-shift assay (shown for bands 1, 3, and 5 in Fig. 2*B*). Under the same conditions, virtually all of the affinity-purified E2F was shifted to a slower-migrating band, as has been reported (8, 24).

Because of the comigration of these proteins on many different chromatography matrices, the dramatic loss of activity following preparative electrophoresis and renaturation, and the inability of the gel-purified E2F components to bind pRB60, it was hypothesized that native E2F might be a complex composed of two or more of the protein components. To test this hypothesis, the five gel-purified fractions were mixed in all possible combinations, refolded, and assayed for E2F activity. A dramatic increase in E2F activity (>100-fold) was obtained with specific combinations compared to the activity of the individual components subjected to the same refolding protocol (Fig. 3*A*). The mobility of the complex reconstituted with bands 1 and 5 is intermediate between those of the individual bands (Fig. 3*B*) and, like all the reconstituted complexes, the band 1 + band 5 complex comigrates with authentic E2F (Fig. 3*A*). These mixing experiments, with the corresponding increase in DNA-binding activity and change in mobility, strongly suggest that the reconstituted complexes bind DNA as heterodimers, while the individual components form homodimers with much lower affinity for the E2F probe. Identical results were obtained with DNA probes containing either a palindromic E2F-binding site (derived from the DHFR promoter) or a

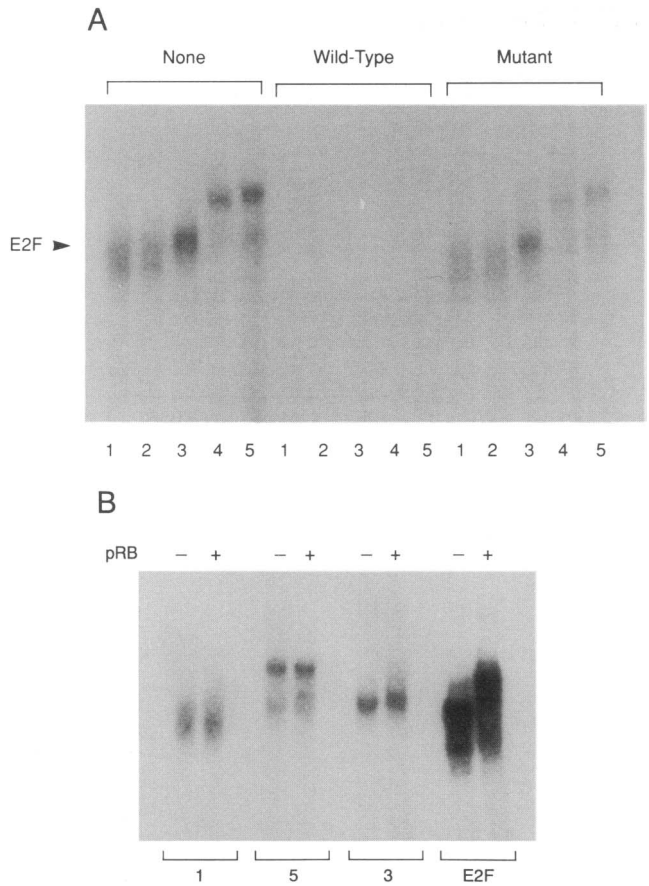

FIG. 2. DNA-binding specificity and pRB binding of individual E2F bands. (*A*) Gel-retardation assay with bands 1–5 (as indicated below lanes) in the absence or presence of a 100-fold excess of competing wild-type or mutant DNA oligomer. Position of affinity-purified free E2F–DNA complex is indicated by arrowhead. (*B*) Gel-retardation assay with bands 1, 3, 5, and affinity-purified E2F in the presence (lanes +) or absence (lanes −) of pRB60 (40 ng/ml).

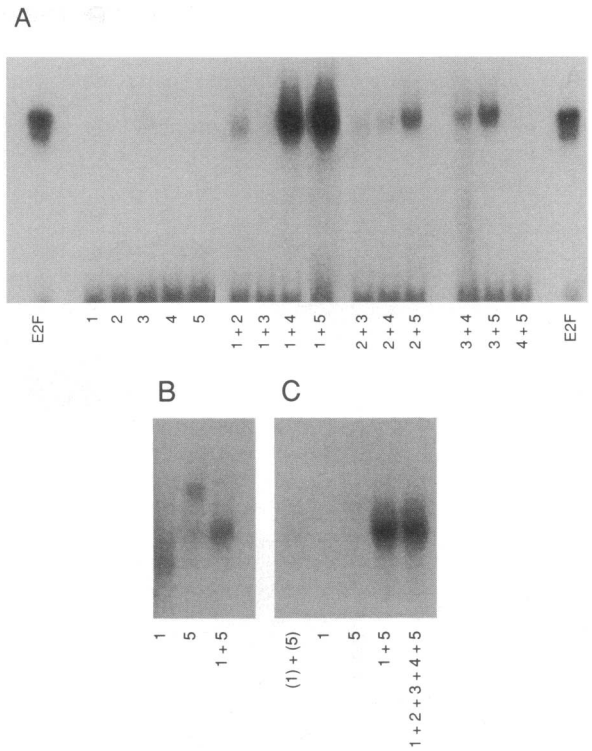

FIG. 3. DNA-binding activity of recombined individual SDS/PAGE-purified E2F proteins in gel-retardation assay. (*A*) Protein bands refolded individually or together (as indicated below lanes) and affinity-purified E2F. (*B*) Comparison of gel-shift mobilities of bands 1 and 5 and the corresponding heterodimer (diluted 1:100). (*C*) Efficiency of heterodimer formation. Bands were refolded individually and combined during the gel-shift assay [lane (1) + (5)] or refolded together (all other lanes). Identical volumes of proteins were used except in last lane (0.5 vol each).

nonpalindromic site (derived from the E2 promoter), suggesting that all of the complexes generated have similar site preferences.

The five individual proteins can be divided into two functional groups (bands 1–3 and bands 4 and 5). Optimal reconstitution of activity required at least one component from each group, with bands 1 and 5 consistently yielding the highest recovery. The E2F activity resulting from mixing bands 1 and 5 is virtually identical to that obtained from mixing all five bands (Fig. 3*C*), suggesting that only two of the fractions are necessary to reconstitute full DNA-binding activity. However, quantitative comparison is complicated by the fact that the reconstituted activity is not linear with the volume of each protein fraction added, despite the large excess of carrier protein present, and that the protein concentration of each fraction is not known. Reconstitution of E2F activity is also critically dependent on co-refolding of the bands. Mixing of individually refolded bands was much less effective at reconstituting E2F activity (Fig. 3*C*).

Recently two laboratories published the sequence of a cloned gene (labeled RBP3 or RBAP1) whose protein product displays many of the properties of authentic E2F (16, 17). To determine whether RBP3 interacts with the gel-purified E2F proteins from HeLa cells, a recombinant N-terminally truncated form of RBP3 containing intact DNA- and pRB-binding domains was purified from *Escherichia coli* as a GST fusion protein. After removal of the GST domain, RBP3-(89–437) was mixed with each of the individual bands, refolded, and tested in the gel-shift assay (Fig. 4*A*). The amount of RBP3 and HeLa proteins in the assay were titrated so that binding of the individual components to the E2F site was minimal. Upon mixing RBP3 with protein from band 1, however, a dramatic increase in DNA binding was observed. Slight stimulation of the DNA binding activity of RBP3 was observed with band 3, and no stimulation resulted from mixing RBP3 with bands 4 and 5. Reconstitution of E2F activity with band 1 and full-length RBP3 resulted in a complex that comigrated with authentic E2F (data not shown). These data demonstrate that RBP3 behaves like bands 4 and 5 of HeLa E2F and suggest that RBP3 may be related to those proteins. This hypothesis is strengthened by the observation that polyclonal anti-RBP3-(409–426) antibodies cross-react with bands 4 and 5, but not with bands 1, 2, and 3, in a Western blot (Fig. 4*C*). Similar results were obtained with polyclonal antisera generated against the full-length RBP3 protein (data not shown). These results also demonstrate that the proteins contained in bands 1, 2, and 3 are immunologically distinct from those of bands 4 and 5.

The observation that RBP3 substitutes for band 5 permits testing of the hypothesis that RBP3 and, by extrapolation, the individual gel-purified E2F components bind DNA as homodimers. This model was tested by generating two forms of RBP3 with different molecular sizes [GST-RBP3-(89–437) and RBP3-(110–191)], which yield gel-shift bands of different mobility (Fig. 4*B*). When combined, these proteins yield a new band in the gel-shift assay with a mobility intermediate between the individual forms, indicating that RBP3 binds the E2F site as a dimer. These results are consistent with a model in which the individual components of HeLa E2F bind DNA

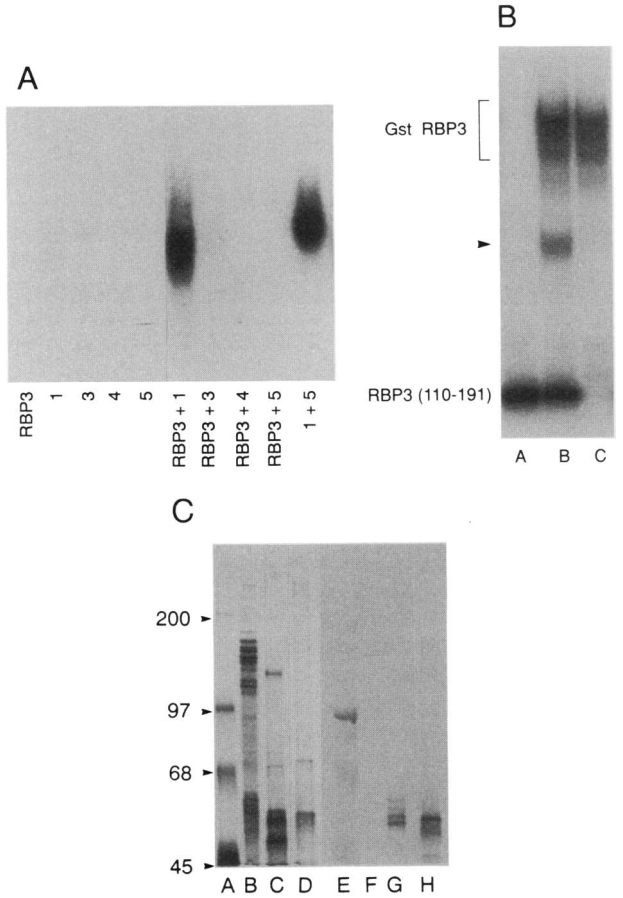

FIG. 4. DNA-binding activity of heterodimers containing RBP3 and cross-reactivity with α-RBP3 antibodies. (*A*) Reconstitution of E2F activity with RBP3-(89–437) and individual E2F bands. Proteins were refolded individually or in combinations (as indicated below lanes). (*B*) Heterodimer formation by RBP3 proteins of different sizes: RBP3-(110–191) (lane A), GST-RBP3-(89–437) (lane C), and a combination of the two proteins (lane B). Proteins at 150 nM each were mixed with each other or buffer A in equal volumes and incubated for 4 hr at 4°C followed by a standard gel-shift assay. Heterodimeric complex is indicated by arrowhead. (*C*) Silver stain (lanes A–D) and Western blot with anti-RBP3-(409–426) IgG (lanes E–H). Molecular size markers (lanes A and E), flow-through of DNA affinity column (lanes B and F), affinity-purified E2F (lanes C and G), and RBP3-(1–437) (lanes D and H). Numbers on left are kDa.

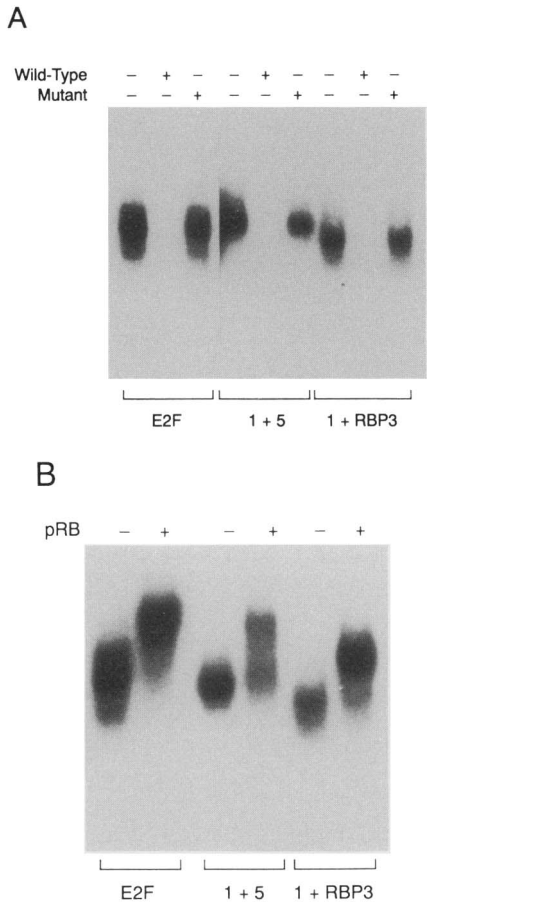

FIG. 5. DNA- and pRB-binding specificities of reconstituted heterodimers. (*A*) Competition of DNA binding of authentic E2F and heterodimers by a 50-fold excess of mutant or wild-type DNA oligomers as indicated. (*B*) Supershifting by pRB105 of E2F–DNA and heterodimer–DNA complexes.

as homodimers, although much less efficiently than as heterodimers.

To verify that the reconstituted heterodimers behave like authentic HeLa cell E2F, the specificities of DNA and pRB binding were compared. The DNA binding of affinity-purified E2F, reconstituted bands 1 + 5, and reconstituted band 1 + RBP3 was not affected by a 50-fold excess of unlabeled mutated E2F probe, demonstrating specific binding by all three complexes (Fig. 5*A*). Unlike the individual homodimers (Fig. 2*B*), the reconstituted heterodimers are supershifted by pRB60 (data not shown) and pRB105 (Fig. 5*B*), as is seen with affinity-purified E2F. Finally, the DNA footprinting patterns resulting from the binding of these proteins to the E2F site are identical (data not shown). These results strongly suggest that authentic E2F binds to DNA as a heterodimer of two distinct proteins.

DISCUSSION

In the current study, we report that E2F purified from HeLa cell lysates appears to bind DNA as a heterodimeric protein. These conclusions are derived from several independent observations. First, multiple proteins with specific E2F DNA-binding activity copurify on a DNA affinity column. Second, while it is possible to separate these proteins by preparative SDS/PAGE, the resulting purified proteins display only weak activity in a gel-shift assay and are not supershifted by pRB60.† Only after mixing and renaturing the proteins are the two key biochemical properties of authentic E2F, high-affinity DNA and pRB binding, regenerated. Third, the involvement of at least two distinct proteins in the E2F activity resulting from the reconstitution experiments is apparent from the intermediate location of the gel-shift band in between the bands obtained from using the SDS/PAGE-purified proteins alone. These results suggest that the individual proteins bind the E2F site as homodimers and that optimal reconstitution of activity in the gel-shift assay requires formation of a heterodimer of intermediate molecular size.

†A recent study by Ray *et al.* (25) suggests that observation of the HeLa E2F/pRB supershifted DNA complex requires the presence of a third protein, RBP60, which is resolved from E2F during E2F purification. Affinity-purified E2F provided by one of the authors of that study (P. Raychaudhuri, University of Illinois, Chicago) behaved exactly like our own E2F in pRB supershift experiments. A supershifted band was apparent under our assay conditions, but E2F DNA binding was apparently inhibited by pRB when assay conditions described by Ray *et al.* (25) were used. We conclude that pRB supershifts the E2F–DNA complex in the absence of RBP60, but that observation of this complex can be masked by the intrinsic DNA-binding activity of pRB if a large excess of nonspecific DNA is included in the assay.

The DNA-binding activity of recombinant RBP3 is consistent with the E2F heterodimer model. Reconstitution of specific DNA-binding activity with RBP3 fragments of different molecular size suggests that RBP3 binds DNA as a dimer. Under the conditions of our gel-shift assay, recombinant RBP3 is not supershifted by pRB (data not shown). pRB binding, as well as high-affinity binding to the E2F site, is observed only when RBP3 is complemented with one of the lower E2F protein components isolated from HeLa cells. RBP3 purified from *E. coli* thus behaves like E2F band 5 from HeLa cells. Further evidence for a close relationship is the cross-reactivity of polyclonal anti-RBP3 antibodies with bands 4 and 5. These same antibodies, however, do not recognize bands 1, 2, and 3, suggesting strongly that the two complementary sets of proteins derived from HeLa cells are not closely related. It is possible, though, that the proteins within each complementation group are differentially phosphorylated or otherwise processed forms of a single gene product.

The observation that E2F displays optimal activity as a heterodimer is consistent with the quaternary structure of many other transcription factors (26). In some cases, homodimer formation is proposed to provide an additional level of transcriptional regulation, with the protein–protein interactions in dynamic equilibrium. The identification of five protein bands with at least weak E2F binding activity allows up to 15 different combinations even in the absence of binding to other cellular proteins, suggesting the potential for a powerful and dynamic regulatory interaction.

RBP3 appears to contain both helix–loop–helix and leucine zipper protein-binding motifs (17), consistent with the finding that it can bind DNA as a dimer. The helix–loop–helix domain contained in RBP3-(110–191) appears sufficient for homodimerization and DNA binding (Fig. 4*B*). E2F and RBP3 homodimers bind the E2F site very weakly, however, and are not supershifted by pRB60. These observations suggest that the optimal DNA- and pRB-binding domains are generated only through contact of the two distinct components of heterodimeric E2F, either through allosteric interactions or by formation of a shared binding site. Since RBP3 can clearly bind pRB60 in immunoprecipitation and blotting assays (refs. 16 and 17; data not shown), it is likely that the homodimers maintain a weak pRB-binding site that is not detected in the gel-shift assay, much as they maintain a weak binding affinity for the E2F site. The influence of the putative leucine zipper and other domains of RBP3 on heterodimerization, high-affinity DNA binding, and complex formation with the cellular components of E2F and other cellular proteins remains to be investigated.

In conclusion, we found that specific individual protein components of the E2F DNA affinity column eluate from HeLa cells exhibit dramatically increased affinity for the E2F site and pRB when they are refolded together in defined combinations. These results are most consistent with the hypothesis that E2F binds DNA as a heterodimer.

The authors gratefully acknowledge Ed Harlow and members of his laboratory for many helpful discussions and for providing RBP3 clones and sequence information prior to publication. We are particularly indebted to Kristian Helin, who first observed that the anti-RBP3-(409–426) antibodies cross-react with our E2F preparation. We would also like to acknowledge Ken Thomas for stimulating discussions.

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