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DNA-binding and trans-activation properties of *Drosophila* E2F and DP proteins

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ABSTRACT **The temporal activation of E2F transcriptional activity appears to be an important component of the mechanisms that prepare mammalian cells for DNA replication. Regulation of E2F activity appears to be a highly complex process, and the dissection of the E2F pathway will be greatly facilitated by the ability to use genetic approaches. We report the isolation of two *Drosophila* genes that can stimulate E2F-dependent transcription in *Drosophila* cells. One of these genes, dE2F, contains three domains that are highly conserved in the human homologs E2F-1, E2F-2, and E2F-3. Interestingly, one of these domains is highly homologous to the retinoblastoma protein (RB)-binding sequences of human E2F genes. The other gene, dDP, is closely related to the human DP-1 and DP-2 genes. We demonstrate that dDP and dE2F interact and cooperate to give sequence-specific DNA binding and optimal trans-activation. These features suggest that endogenous *Drosophila* E2F, like human E2F, may be composed of heterodimers and may be regulated by RB-like proteins. The isolation of these genes will provide important reagents for the genetic analysis of the E2F pathway.**

Several lines of evidence indicate that the transcription factor E2F coordinates the expression of genes during the progression from the G_1 phase of the cell cycle into S phase. E2F-binding sites have been identified in the promoters of several cellular genes whose products are required for cell proliferation (for a review, see refs. 1 and 2). In the c-*myc*, dihydrofolate reductase, c-*myb*, thymidine kinase, and *cdc2* promoters the E2F sites have been shown to be essential for the transcriptional activation of these genes that occurs as serum-starved cells are stimulated to progress through the cell cycle (3–8). In this experimental system, a short element carrying two overlapping E2F sites has been shown to be sufficient for the temporal expression of the dihydrofolate reductase gene at the G_1-to-S transition (9).

Human E2F is a heterogeneous factor representing the combined activity of many different gene products. Four genes have been demonstrated to encode components of E2F called E2F-1, E2F-2, E2F-3, and DP-1, and it is likely that additional genes exist (10–14). Using partially purified human E2F, Huber *et al.* (15) found that specific binding to E2F sites was greatly stimulated when different E2F fractions were combined. E2F-1 and DP-1 associate into stable complexes and activate transcription in a cooperative manner (16, 17). It is not known how many different E2F complexes exist or whether these multiple forms of E2F are functionally redundant.

E2F-dependent transcription of several promoters has been found to be repressed in cells that lack the retinoblastoma protein (RB) by the overexpression of either the RB gene or the related p107 gene (18–24). RB and p107 both associate with E2F *in vivo* and inhibit E2F by directly binding to a region of the protein important for trans-activation (refs. 17 and 24; B.D.D. and E. Harlow, unpublished results). RB, p107, and a homologous protein, p130 (25), have been detected in DNA-bound E2F complexes at various points in the cell cycle. The p107/E2F and p130/E2F complexes also contain cyclins (A or E) and Cdk2 and carry a potent kinase activity (25–32). The temporal appearance of these E2F complexes suggests that they each provide different elements of E2F regulation, but the precise roles of these complexes are unknown.

To date, studies of E2F have relied primarily on a biochemical approach. However, investigations into the overall role of E2F in the control of proliferation and a determination of the functions of the E2F regulatory complexes will require a combination of biochemical and genetic approaches. As the scope of genetic experiments in mammalian cells is limited, we wished to determine whether the E2F-containing growth control pathway could be found in an organism that is more genetically tractable. Here we report the isolation of two *Drosophila* genes encoding E2F-related proteins and demonstrate that they share structural and functional characteristics with their human counterparts.†

MATERIALS AND METHODS

Library Screening and Isolation of cDNAs. One million plaques from a *Drosophila* λgt10 cDNA library (33) were screened with DNA fragments that corresponded to the regions encoding aa 89–214 of E2F-1, aa 85–200 of E2F-2, and aa 132–270 of E2F-3. The probes were labeled with [α-^{32}P]dCTP by random primer extension (34) and used in low-stringency hybridization. Positive clones were plaque purified and cDNA inserts were subcloned into pBluescript SK (Stratagene) for sequencing. The inserts were digested with exonuclease III and S1 nuclease to generate nested sets of deletions, which were sequenced with Sequenase 2.0 (United States Biochemical). To isolate dDP, the same library was screened with a probe to the putative DNA-binding domain of DP-1 (17).

Plasmids. pBS-dE2F was made by subcloning a 4.4-kb *Eco*RI fragment (the entire cDNA) from λ phage 16 into pBluescript SK(+). pBS-dE2F.ATG is a modified form of pBS-dE2F constructed by use of PCR to delete the first 849 bp of pBS-dE2F. pBS-dDP was made by subcloning the entire cDNA insert on an *Eco*RI fragment from λ phage 3 into *Eco*RI-cut pBluescript SK(+). The expression plasmid Act-PPA, the internal control plasmid copia-lacZ (35), and the chloramphenicol acetyltransferase (CAT) reporter $(E2F)_4$-BCAT (17) used in transfection experiments have been described. Act-dE2F and Act-dDP contain the entire coding regions of the genes.

Cell Culture and Transfections. Schneider line 2 (SL2) cells (36) (generously supplied by Jayne Kassel, Massachusetts General Hospital Cancer Center, Boston) were maintained at room temperature in Schneider's *Drosophila* medium and

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Abbreviations: GST, glutathione *S*-transferase; CAT, chloramphenicol acetyltransferase.

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†The sequences reported in this paper have been deposited in the GenBank data base (accession nos. X78421 and X79708).

were transfected by a calcium phosphate method (37). Various amounts (0.2–5 μg) of Act-dE2F and Act-dDP expression plasmids were transfected along with 1 μg of copia-lacZ and 5 μg of $(E2F)_4$BCAT. Transfection mixes were supplemented with pBluescript SK(+) to bring the total amount of DNA transfected up to 20 μg per 10-cm plate, and the total amount of expression vector added was kept constant by the addition of the expression vector Act-PPA, which lacked an insert. Cell harvesting and extract preparation and treatment were as described (37) except that cells were lysed by three freeze–thaw cycles. CAT assays were performed by the liquid scintillation method of Sleigh (38). β-Galactosidase was assayed as described (35). Each transfection was performed at least four times independently (twice in duplicate), and representative data are shown.

***In Vitro* Binding Assays.** dDP and dE2F cRNAs were synthesized from the linearized cDNAs by *in vitro* transcription and translated in rabbit reticulocyte lysate (Promega) in the presence of [^{35}S]methionine (New England Nuclear). The synthesized polypeptides were then incubated for 60 min with glutathione-agarose-bound glutathione *S*-transferase (GST) fusion protein in 200 μl of ELB$^+$ buffer (10). The complexes were washed four times with ELB$^+$ and then resolved by SDS/10% PAGE.

GST fusion proteins were prepared by amplification with Vent polymerase (New England Biolabs) using the appropriate primers with *Bam*HI or *Eco*RI restriction sites at their 5′ ends. The amplified fragments were digested with *Bam*HI and *Eco*RI and subcloned into pGEX-2T (Pharmacia); the GST fusion proteins were expressed and purified from *Escherichia coli* as described (39). After binding to glutathione-agarose, the size, purity, and concentration of the GST fusion proteins were evaluated by Coomassie blue staining of SDS/polyacrylamide gels. Approximately 2 μg of full-length protein was added to each binding reaction mixture.

For DNA-binding assays, labeled polypeptides were synthesized by *in vitro* translation, diluted with 100 μl of ELB$^+$ buffer, and incubated with 50 μl of Sepharose 4B beads that had been coupled with either wild-type or mutant E2F oligonucleotides. The slurry was rocked for 60 min at 4°C to allow binding to occur. The beads were washed four times in ELB$^+$, and the bound proteins were analyzed.

RESULTS

Isolation of cDNAs Encoding dE2F. We prepared probes that spanned the homologous DNA-binding domains of E2F-1, E2F-2, and E2F-3 and used this mixture to screen a *Drosophila* λgt10 cDNA library. The library used had been constructed from larval eye imaginal discs (33), a tissue containing both growing and quiescent cells. Multiple clones were identified, purified, and analyzed. The inserts varied from 1.8 to 4.4 kb. The largest had a single long open reading frame that predicted a protein of 805 aa (Fig. 1*A*). The longest cDNA clone contained a relatively long 5′ untranslated sequence that had multiple in-frame termination codons upstream of the putative initiator methionine (data not shown). During the course of this work, another laboratory independently isolated an E2F-related cDNA from *Drosophila* (40), and comparison of the two sequences revealed that their cDNA and the one reported here were derived from the same gene and were identical throughout their coding regions. A BLAST database search (April 1994) and use of protein-alignment programs indicated significant similarity with the human proteins encoding the E2F family of protein (Fig. 1*B*). Although the fly and human proteins share three regions with a high percentage of homology, the *Drosophila* protein is much larger in size, in part due to a 300-aa insertion of unique sequence between the two most C-terminal blocks of homology. The fly gene appears to be equally related to

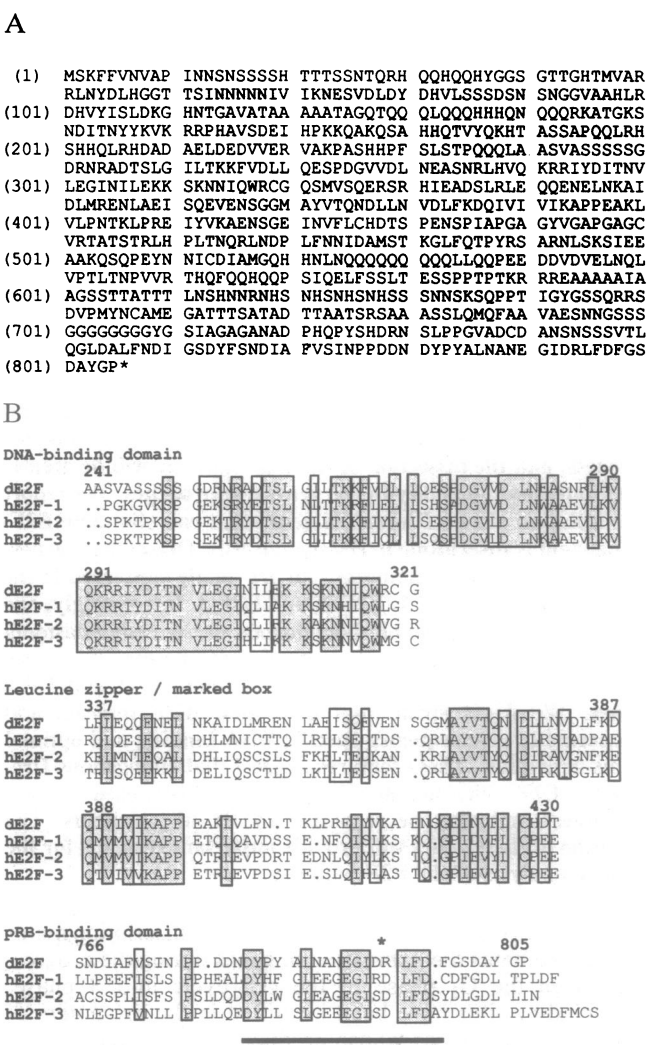

FIG. 1. (*A*) Predicted amino acid sequence of dE2F. Star indicates termination codon. (*B*) An alignment of the three regions most conserved between dE2F and three members of the human E2F family of proteins (10–13). Numbering above the amino acid sequence corresponds to the *Drosophila* E2F sequence, and dots indicate gaps introduced for sequence alignment. Boxed residues indicate amino acids that are similar in fly E2F and all three human proteins, and shaded boxes indicate residues that are identical in all four proteins. Stringent similarity rules were used: Q, N; V, I, L; S, T; K, R; and D, E. The bar indicates the previously defined minimal RB-binding domain in human E2F-1, and the star denotes the single residue within this domain that is identical in all three human proteins but differs in dE2F.

each of the human E2F family members (13). To reflect this fact, we have termed this protein dE2F.

aa 249–318 of dE2F share striking homology (Fig. 1*B*) with DNA-binding domains of human E2F genes. In addition, a region termed the "marked box" (13) that is highly conserved between the human proteins is also conserved within dE2F. The function of this domain is uncertain but may be involved in protein dimerization (17). The RB-binding domain has also been largely conserved in dE2F, sharing 56% similarity over this region (Fig. 1*B*). dE2F contains numerous repetitive amino acid stretches including poly(glutamine) segments reminiscent of those found in several mammalian and *Drosophila* transcription factors, such as Sp1 (37) and NTF-1 (41). Similar repetitive sequences are encoded in a large number of *Drosophila* genes. Consistent with this, DNA probes prepared from the full-length dE2F cDNA hybridized to multiple DNA fragments on Southern blots of genomic

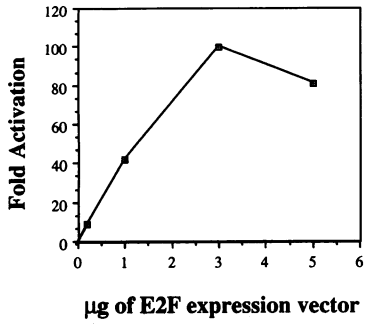

FIG. 2. Trans-activation by dE2F expressed in insect cells. From 0.2 to 5 μg of dE2F expression vector was transfected into *Drosophila* SL2 cells along with an E2F-responsive CAT reporter gene [$(E2F)_4$BCAT]. Fold activation is the ratio of CAT activity to β-galactosidase activity expressed from the internal control plasmid copia-lacZ. The activity of the internal control plasmid was not affected by the expression vectors used here or in Fig. 5.

DNA even at high stringency (data not shown). These fragments do not appear to represent E2F-related genes, since a probe prepared from the putative DNA-binding domain of dE2F, containing sequences that are most likely to be conserved in a homologous gene, produced a single strong hybridization signal even at low stringency (data not shown). We conclude that the dE2F gene is a single-copy gene and is not a member of a large family of genes that can be readily detected by cross-hybridization.

Transcriptional Activation Properties of dE2F. We tested whether the expression of dE2F in insect cells could promote transcriptional activation of an E2F-dependent promoter, since this functional assay represents the most rigorous test of a putative E2F gene. For this assay, dE2F expression was driven from the *Drosophila* actin 5C promoter. This vector was cotransfected into the *Drosophila* SL2 cells (36) together with a CAT reporter gene [$(E2F)_4$BCAT (17)] containing four consensus E2F binding sites (Fig. 2). Neither the parent reporter plasmid [BCAT, which contains only a TATA box (42)] nor the internal control plasmid [copia-lacZ (35)] was affected by the expression of dE2F (data not shown and Fig. 2). Expression of dE2F led to a significant increase in transcriptional activity of the reporter, with optimal activation levels approaching 100-fold, indicating that dE2F is a bona fide homolog of the human E2F genes.

Isolation of cDNAs Encoding dDP. In light of the transcriptional activation properties of dE2F we were surprised to find that recombinant dE2F synthesized by *in vitro* translation was unable to bind to oligonucleotides containing E2F sites (data not shown; see also Fig. 5*A*). As the DNA-binding and trans-activation properties of human E2F proteins are greatly enhanced by a heterodimeric partner (15–17), we hypothesized that the SL2 cells contained such a partner for dE2F. The most likely candidate for such an activity would be the product of a homolog of the mammalian DP-1 genes (14), and we therefore screened the *Drosophila* cDNA library with a labeled fragment corresponding to the putative DNA-binding domain of mouse DP-1. DNA sequencing of cDNAs from four independent positive phage revealed that the inserts contained overlapping sequences. The longest cDNA clone (2.1 kb) was completely sequenced and found to contain a long open reading frame of 377 aa (Fig. 3). The *Drosophila* and human DP-1 proteins share 61% identity over a region between residues 91 and 315 that includes the putative DNA-binding domain (Fig. 3). The *Drosophila* gene appears to be equally related to both human DP-1 and a highly related gene, human DP-2, that has been isolated recently by hybridization with a DP-1 probe (C.-L. Wu, personal communication). It is unclear whether the *Drosophila* gene is the homolog of either one or both of the human genes; thus, we have termed the *Drosophila* gene dDP. Genomic Southern blots of *Drosophila* DNA were probed under low and high stringency with the entire dDP cDNA. Under both sets of conditions, only a single strongly hybridizing fragment was seen (data not shown). Although it appears that dDP is encoded by a single gene in *Drosophila*, we cannot rule out the possibility that other, more distantly related genes exist in the fly.

dE2F and dDP Associate *in Vitro*. dDP and dE2F were expressed as GST fusion proteins in bacteria and tested for binding to [^{35}S]methionine-labeled proteins produced by *in vitro* transcription and translation of the cDNA clones. Labeled dE2F bound strongly to GST–dDP but failed to bind to GST–dE2F controls (Fig. 4*A*). In the converse experiment, labeled dDP bound to GST–dE2F, and deletion mutants of

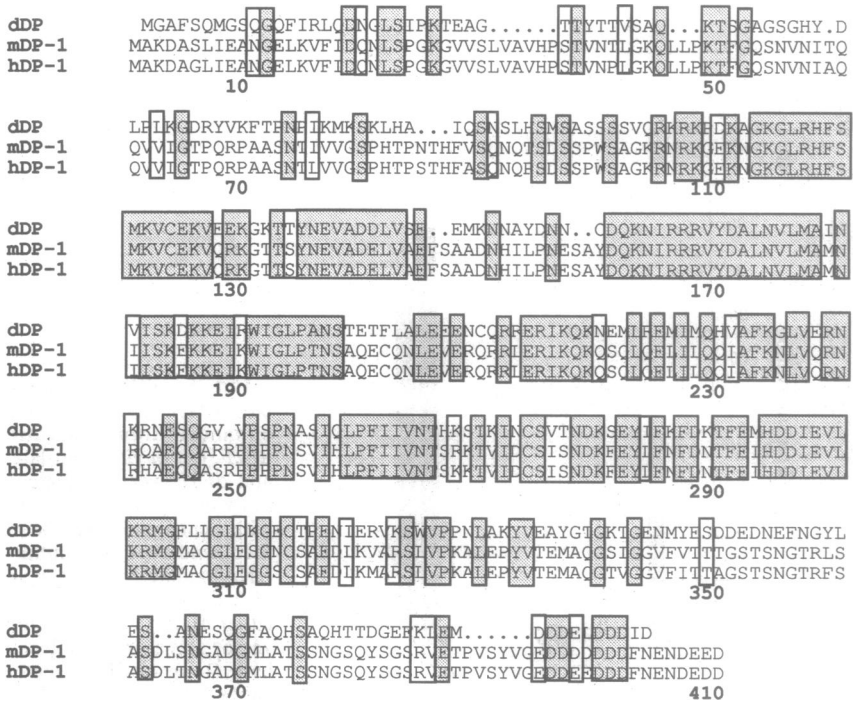

FIG. 3. Predicted amino acid sequence of dDP aligned with human (h) and mouse (m) homologs (14, 17). Numbering corresponds to the mouse and human amino acid sequence, and dots represent gaps introduced to allow the best alignment. Residues are boxed and shaded as described in the legend of Fig. 1. Similarity rules are described in Fig. 1.

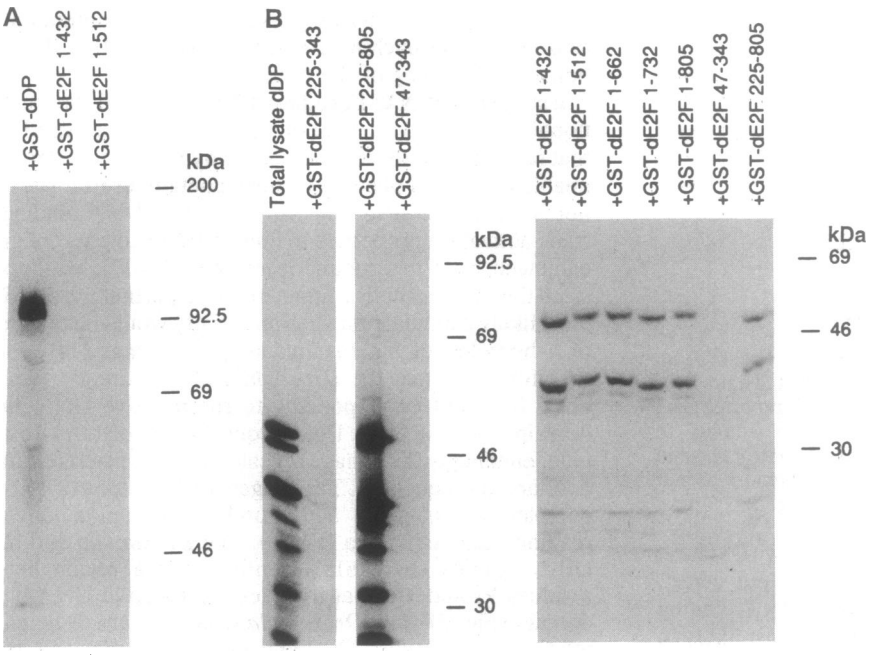

FIG. 4. dE2F and dDP associate *in vitro*. Binding assays were performed using GST fusion proteins and dE2F or dDP synthesized by *in vitro* transcription of the cDNA clone and translation using [^{35}S]methionine. GST fusion proteins containing full-length dDP, dE2F, and deletion mutants were tested for binding. The amino acids present in the dE2F fusion proteins are indicated. (*A*) *In vitro* translated dE2F binds to GST–dDP. Two GST-dE2F deletion mutants were included as negative controls. (*B*) *In vitro* translated dDP binds to GST–dE2F and to several dE2F deletion mutants.

dE2F were constructed to identify the regions important for this interaction (Fig. 4*B*). dDP bound to dE2F fusion proteins containing aa 225–805 or 1–432 but failed to bind to a fusion protein that included only aa 47–343. These data suggest that a region of dE2F between aa 225 and 432 may be required for interaction with dDP. Interestingly, this region of the protein is highly conserved between *Drosophila* E2F and human E2F-1, -2, and -3 (Fig. 1*B*), and an overlapping portion of human E2F-1 has been implicated in binding human DP-1 (17).

DNA-Binding and Trans-Activation Properties of dDP. Next, we tested the ability of recombinant dDP or dE2F to bind DNA, either alone or in combination. Labeled proteins were generated by *in vitro* translation and incubated with oligonucleotides bearing either wild-type or mutant E2F binding sites (Fig. 5*A*). In these experiments, dDP appeared to have a weak nonspecific DNA-binding activity; dE2F alone was unable to bind wild-type or mutant E2F DNA sequences. Interestingly, when dDP and dE2F were mixed, dE2F acquired DNA-binding activity, and the affinity of dDP for DNA was dramatically enhanced. The enhanced binding by these proteins was site-specific and was observed with wild-type but not mutant E2F sites. These data, together with the fact that dDP and dE2F can associate, suggest that dDP promotes the sequence-specific DNA-binding of dE2F and that this binding is achieved through the interaction of these two proteins.

Given that dDP was able to bind DNA weakly and stimulate the DNA-binding ability of dE2F, we investigated the effect of dDP expression on the transcriptional activity of an E2F-responsive promoter. Hence, we performed transient transfection experiments in SL2 cells, using either dDP alone

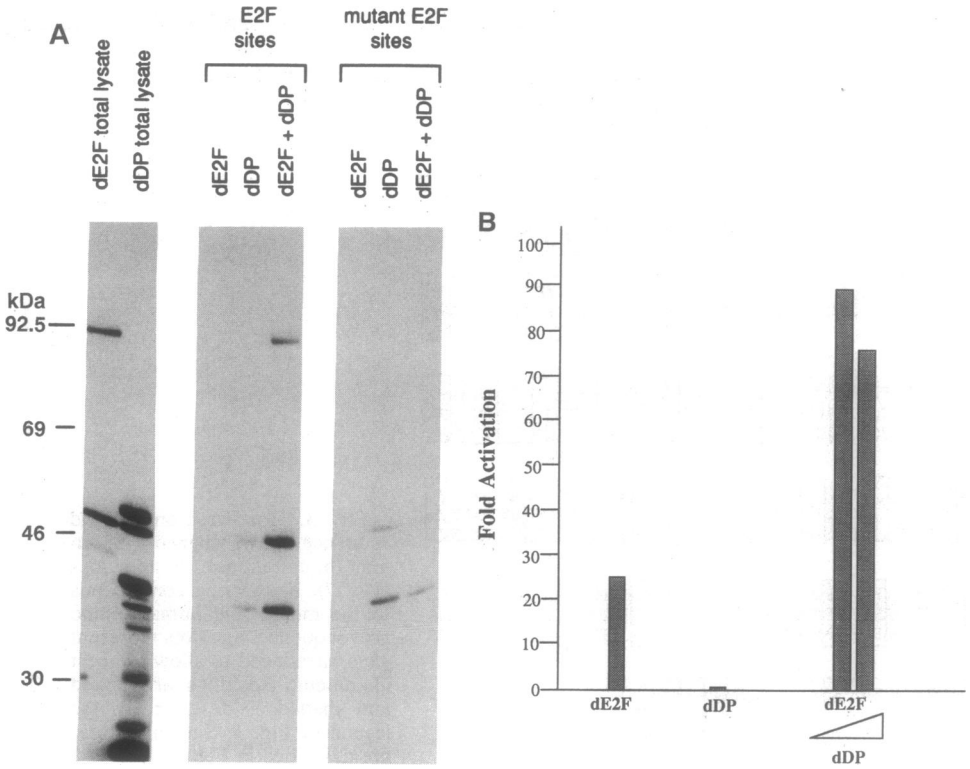

FIG. 5. dDP potentiates specific DNA binding by dE2F and enhances trans-activation by dE2F. (*A*) *In vitro* translated dDP and dE2F were incubated either individually or in combination with Sepharose beads bearing either wild-type or mutant E2F DNA binding sites. (*B*) *Drosophila* SL2 cells were transfected with either dE2F (1 μg) or dDP (1 μg) vector alone or a combination of both (1 μg of dE2F vector with either 0.2 or 1 μg of dDP vector).

or in combination with dE2F as described earlier. Interestingly, overexpression of dDP alone had no effect on the level of transcription from the E2F-containing promoter (Fig. 5*B*). However, when dDP and dE2F were coexpressed, the combination of proteins resulted in a level of activation considerably greater than that seen with dE2F alone (Fig. 5*B*). The magnitude of the effect of dDP enhancement depended on the amount of dE2F plasmid used. At subsaturating amounts of dE2F plasmid (1 μg in Fig. 5*B*), coexpression of dDP increased activation from 25- to 90-fold. A greater level of enhancement was observed when less dE2F plasmid was used or when dE2F and dDP were expressed in these insect cells using a weaker promoter (data not shown). We conclude from these experiments that dE2F and dDP interact productively to form a heteromeric complex capable of site-specific DNA binding and potent transcriptional activation.

DISCUSSION

The two *Drosophila* genes reported here, dE2F and dDP, encode proteins with many of the characteristics of the human E2F genes. The dDP amino acid sequence is highly homologous to human DP-1 and DP-2 genes, and dE2F contains three regions that are conserved in human E2F genes. dDP and dE2F bind together and act cooperatively to stimulate sequence-specific DNA binding and site-specific trans-activation. Thus the dE2F and dDP genes are bona fide homologs of their mammalian counterparts, since they conserve both the structural and the functional properties of the human genes. As such, they will serve as the focal point for a genetic study aimed at examining the regulators and targets of E2F activity.

Southern blot analysis of *Drosophila* DNA, however, failed to detect genes closely related to dE2F or dDP. This suggests that flies have bypassed the need for a large family of E2F-1-related polypeptides. Further studies will be needed to determine whether there are related genes that are too divergent to be found by cross-hybridization. If no additional genes are found this may limit the parallels that can be drawn between the regulation of E2F activity in human and *Drosophila* cells. It appears, however, that at least one potential target of dE2F and dDP has been conserved between flies and humans: the *Drosophila* DNA polymerase α gene, like its human counterpart, contains multiple E2F-binding sites which can confer dE2F responsiveness on a heterologous reporter gene (40).

The question of the existence of an E2F pathway in *Drosophila* was of particular interest for several reasons. (*i*) In mammalian cells, E2F activity has been shown to be downregulated during differentiation and implicated as a regulator of genes necessary for cellular proliferation (1–8, 43). (*ii*) E2F appears to provide a direct connection between cyclin-dependent kinases and the regulation of specific transcriptional events. (*iii*) E2F-dependent transcription is inhibited by RB, the product of a known tumor-suppressor gene. In some settings, RB itself has cell cycle-regulatory properties and may provide a direct link to cell cycle-regulatory kinases. Although it would be useful to study RB in *Drosophila*, no homolog has been found by direct screening procedures. Within the minimal RB-binding domain of E2F-1, 9 of the 10 aa conserved among all three human E2Fs are also identical in the dE2F sequence (Fig. 1*B*). This striking similarity suggests that the regulatory interaction between RB-related proteins and E2F activity may be conserved in *Drosophila*, and a genetic analysis of E2F activity in *Drosophila* might permit the isolation of homologs for RB or RB-related proteins.

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