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Functional Properties of a *Drosophila* Homolog of the E2F1 Gene

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A variety of studies have now implicated the cellular transcription factor E2F as a key participant in transcription control during the cell growth cycle. Although the recent isolation of molecular clones encoding proteins that are components of the E2F activity (E2F1 and DP-1) provides an approach to defining the specific involvement of E2F in these events, definitive experiments remain difficult in the absence of appropriate genetic systems. We have now identified a *Drosophila* equivalent of E2F1 that we hope will allow an eventual genetic approach to the role of E2F in cellular regulatory events. A cDNA clone was isolated from a *Drosophila* cDNA library by using a probe containing sequence from the E2F1 DNA binding domain. The sequence of the clone, which we term drosE2F1, demonstrates considerable homology to the human E2F1 sequence, with over 65% identity in the DNA binding region and 50% identity in the region of E2F1 known to interact with the retinoblastoma gene product. A glutathione *S*-transferase–drosE2F1 fusion protein was capable of binding specifically to an E2F recognition site, and transfection assays demonstrated that the drosE2F1 product was capable of transcription activation, dependent on functional E2F sites as well as sequences within the C terminus of the protein. Finally, we have also identified E2F recognition sequences within the promoter of the *Drosophila* DNA polymerase α gene, and we demonstrate that the drosE2F1 product activates transcription of a test gene under the control of this promoter. We conclude that the drosE2F1 cDNA encodes an activity with extensive structural and functional similarity to the human E2F1 protein.

The E2F transcription factor was originally identified as a cellular factor involved in the adenovirus E1A-mediated activation of the viral E2 gene (25). Subsequent studies have shown that E2F is normally complexed to a variety of cellular proteins, that these interactions prevent the utilization of E2F for viral E2 transcription, and that the E1A protein is capable of disrupting these E2F complexes, releasing an E2F that is transcriptionally active (1). The proteins in association with E2F include the retinoblastoma gene product (Rb), the Rb-related protein p107, and the cell cycle regulatory proteins cyclin A, cyclin E, and the cdk2 kinase (2, 4–7, 11, 26, 31, 35). Most of these proteins were previously identified as targets for the action of E1A (39) as well as simian virus 40 T antigen (10) and the E7 product of human papillomaviruses (15). It would appear that the binding of E1A, T antigen, or E7 to Rb, as well as to the other proteins, reflects the capacity of these viral proteins to disrupt the E2F complexes (30).

The significance of these findings derives not only from the identification of a common activity of the viral oncoproteins but also from the realization that E2F is a potential cellular target for important cellular growth-regulating activities, including the Rb tumor suppressor gene product. Indeed, a series of experiments has now provided convincing evidence for the interaction of Rb with E2F as an important event in the function of Rb as a growth-suppressing protein, since there is a direct correlation between (i) the ability of Rb to interact with E2F and inhibit the transcriptional activating capacity of E2F (17, 20, 38) and (ii) the ability of Rb to suppress cell growth (32, 33). In short, it appears that the E2F transcription factor may play a key role in cellular growth control.

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The identification of cellular genes that appear to be regulated by E2F offers clues as to the specific role that E2F plays in cellular growth control. In particular, a group of genes that are commonly regulated in late G_1 of the growth response and that encode activities important for cellular DNA replication appear to be regulated by E2F. Genes such as those encoding dihydrofolate reductase (DHFR), thymidine kinase, and DNA polymerase α contain E2F sites within essential promoter sequences (30), and at least for the DHFR gene, these E2F sites are critical for regulated expression in late G_1 (36).

The recent isolation of cDNA clones that encode proteins with E2F activity (16, 18, 24, 34) has now provided an approach to a further, more complete understanding of the role of E2F in cellular regulatory processes. Analyses of these clones have identified domains important for the DNA binding capacity of the protein as well as the ability to stimulate transcription. Nevertheless, given the lack of a straightforward genetic system in which to explore the role of E2F1, or other E2F activities, further assays will prove to be difficult. Although it is now possible to disrupt a gene such as that encoding E2F1 in the mouse genome and then assess the consequences of a loss of function, this is a laborious approach and will yield limited information should E2F1 prove to be essential for cell viability. In addition, the mouse is not a facile experimental system in which to genetically identify interacting components.

In view of these limitations and concerns, we have attempted to identify related activities in organisms that do offer a genetic approach to the determination of cellular function. Given the potential role of E2F in cell cycle control, the organism of choice would be a yeast because of the rich body of information that has accumulated from the combined approaches of genetics and biochemistry. Nevertheless, we have been unable to identify an E2F homolog in either budding yeast or fission yeast cells. We have, however, been successful in identifying an E2F1 equivalent in *Drosophila melanogaster*. A variety of

analyses suggest that the product of this gene, which we term drosE2F1, exhibits extensive similarity to the human E2F1 gene product.

MATERIALS AND METHODS

Southern blot assays. Cellular DNA was digested with restriction endonucleases, electrophoresed in a 0.7% agarose gel, and blotted onto a nitrocellulose membrane. The membrane was baked at 80°C for 2 h and then probed with ^{32}P-labeled fragments of E2F1 cDNA. Hybridization was carried out in a solution of 5X SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris-HC1 (pH 7.5), 5× Denhardt's solution, 100 μg of heat-denatured sheared salmon sperm DNA per ml, and 45% formamide for the 5′ half of the E2F1 cDNA probe (*Bam*HI-*Bgl*II fragment of E2F1 cDNA from pGEX-2TK-RBAP-1 [24]) and 35% formamide for the 3′ half of the E2F1 cDNA probe (*Bgl*II-*Eco*RI fragment from pGEX-2TK-RBAP-1) at 42°C overnight. The membranes were washed three times with 0.5× SSC–0.1% SDS at 60°C for the 5′-half probe and 2× SSC–0.1% SDS at 50°C for the 3′-half probe. Filters were exposed to X-ray films with intensifying screens at −70°C.

Screening of a *Drosophila* cDNA library. A λgt10 *Drosophila* cDNA library prepared from poly(A)$^+$ RNA from total imaginal discs was screened with the *Bam*HI-*Bgl*II fragment of E2F1 cDNA in the same conditions as for Southern blotting. Positive clones identified as overlapping signals in duplicate filters were picked and subjected to a second round of screening. Reproducibly positive clones were further subjected to tertiary screening to obtain single clones. The isolated phage were amplified by the plate lysate method, and inserted cDNA fragments were cut out and subcloned into plasmid pUC18. The identities of the clones was examined by restriction mapping experiments using *Hae*III, *Sau*3AI, and *Dde*I and by sequencing of both ends of all cDNA clones.

DNA sequencing. Both strands of the largest insert of the major group were sequenced. A series of unidirectional deletions was generated by using the Erase-a-Base system kit (Promega Corp.) according to the manufacturer's procedure. Double-stranded DNA sequencing was performed with a Sequenase version 2.0 kit (United State Biochemical Corp.) with the protocol provided by the manufacturer.

Construction of plasmids. pGEX-drosE2F1 was generated by subcloning of an *Nae*I fragment (nucleotides [nt] 1372 to 2021) into the T4 polymerase blunt-ended *Bam*HI site of pGEX-2T in frame. This plasmid expresses amino acids (aa) 224 to 440 of drosE1F1 fused to glutathione *S*-transferase (GST) protein sequences. A cytomegalovirus promoter-driven drosE2F1 expression vector was generated by insertion of the entire *Eco*RI fragment from the λgt10 clone into the *Eco*RI site of pCMV5 in the sense orientation. A *Drosophila* actin 5C promoter-driven drosE2F1 expression vector was generated as follows. First, a *Sal*I fragment (nt 927 to 1397) of drosE2F1 cDNA was inserted into the *Xho*I site of pPac (8) in the sense orientation. Second, the 5′ *Apa*I site (nt 3835) of drosE2F1 cDNA was changed to an *Xba*I site, and an *Nhe*I (nt 1272)-*Xba*I (nt 3835) fragment was cut out and inserted into an *Nhe*I site in the *Sal*I fragment, restoring the *Nhe*I site. The resultant plasmid, pdrosE2F1$_{WT}$, expresses the drosE2F1 protein (aa 77 to the C-terminal end) fused to the N-terminal 11 aa of the *Ubx* gene product. C-terminal deletion mutants of the plasmid (pdrosE2F1$_{dl560}$ and pdrosE2F1$_{dl769}$) were generated by inserting a reading frame termination linker (CTAGCTAGC TAG) into the *Bsp*EI site (nt 2377) and 3′ *Eco*RV site (nt 3005), respectively. *Drosophila* actin 5C promoter-driven GAL4-drosE2F1 fusion expression vectors were constructed as follows. The GAL4 DNA binding domain (aa 12 to 147) and multiple cloning sites were cut from pSG424 by *Sph*I and *Xba*I, blunt ended by T4 polymerase treatment, and inserted into the T4 polymerase blunt-ended *Xho*I site of plasmid pPac in frame, generating pGal. The GAL4 binding domain expressed from this plasmid lacks the N-terminal 11 aa and is fused to the N-terminal 11 aa of the *Ubx* gene product. The *Apa*I site (nt 3835) of drosE2F1 cDNA was changed to a *Sac*I site, and *Bsp*EI (nt 2377) and 3′ *Eco*RV (nt 3005) sites were changed to *Kpn*I sites by inserting *Kpn*I linkers. Each *Kpn*I-*Sac*I fragment was cut out and inserted into *Kpn*I-*Sac*I sites of the multiple cloning sites of pGal in frame, generating pGal/dE2F1$_{560–805}$ and pGal/dE2F1$_{769–805}$, respectively. These plasmids express the drosE2F1 protein from aa 560 and 769, respectively, to the C-terminal end fused to the GAL4 DNA binding domain. An E2 promoter-driven chloramphenicol acetyltransferase (CAT) plasmid possessing four copies of the E2 enhancer and its mutant, pE2WTx4CAT and pE2MTx4CAT, were generated by insertion of oligonucleotides corresponding to −69 to −34 of the E2 promoter and a mutant version (22) into the *Bgl*II site of p3ME2CAT (28) in a tandemly repeated form in the sense orientation. The *Drosophila* DNA polymerase α promoter-driven CAT plasmid pdPolαCAT was made by insertion of a PCR-amplified genomic DNA fragment spanning from −600 to +42 of the *Drosophila* DNA polymerase α gene into pCAT3M (28). *Drosophila* genomic DNA was PCR amplified by using 5′ (GATCTTCTAGAGCATGATAAATAAAATA CGATT) and 3′ (GACTGAGATCTCAGTTTGCAAAAAT TGT) primers flanked by *Xba*I and *Bgl*II restriction sites, respectively, digested by *Xba*I and *Bgl*II, and subcloned into the *Xba*I-*Bgl*II sites of pCAT3M.

DNA binding assays. Gel mobility shift assays were performed essentially as described previously (40), using an *Eco*RI-*Hin*dIII fragment containing two E2F recognition sites from the ATF(−)adenovirus E2 promoter plasmid (28). Competitors used in binding assays were oligonucleotides corresponding to −69 to −34 of the E2 promoter and a mutant form of this sequence (22). GST fusion proteins were expressed in *Escherichia coli* BL21 and affinity purified by using glutathione-Sepharose 4B (Pharmacia) as described previously (37). The amounts of GST-drosE2F1 protein used were 250 ng and 1 μg per reaction, and 300 ng of GST-E2F1 was used as a positive control. For potential E2F recognition sites in the *Drosophila* DNA polymerase α promoter, chemically synthesized oligonucleotides and *Drosophila* Schneider cell nuclear extract were used. The sequences of double-stranded oligonucleotides were as follows:

1, tccTCGGATTTCCCGCCAAAATATA
GCCTAAAGGGCGGTTTTATATagga

2, tccGATATGTTCCCGCCATTC
TATACAAGGGCGGTAAGaggc

3, tccCCGCCATTCCCGCTTTGA
GCGGTAAGGGCGAAACTaggg

4, tccCTGAAGCTCGAAAATGTA
ACTTCGAGCTTTTACATaggg

Nuclear extract from Schneider cells was prepared as described previously (12). Oligonucleotide 1 was used as a probe. A 100-fold molar excess of oligonucleotide 1 and the wild-type and mutant E2 oligonucleotides and a 500-fold molar excess of oligonucleotides 1 to 4 were used as competitors. A rabbit

polyclonal anti-drosE2F1 antibody raised against the GST-drosE2F1 fusion protein was used at a 1:320 dilution.

CAT assays. Plasmid DNA transfection into the T98G human glioblastoma cell line was performed as described previously (9). Increasing amounts of pCMV-drosE2F1 (100 ng, 500 ng, and 2.5 μg) were cotransfected with 5 μg of pE2WTx4CAT as a reporter plasmid, 5 μg of pRSV-β-gal (29) as an internal control, and 10 μg of carrier DNA (Specialty Media, Inc.); 100 ng of pCMV-E2F1 (9) was used as a positive control. The total amount of expression vector was adjusted by addition of the empty vector. The *D. melanogaster* Schneider II cell line was cultured and transfected essentially as described previously (13). pPac, pdrosE2F1wt, and its deletion mutants (pdrosE2F1dl560 and -dl769) (100 ng of each) were cotransfected with 5 μg of pE2WTx4CAT and pE2MTx4CAT or pE2WTCAT and pE2CAT(E2F−) (28) as reporter plasmids and 10 μg of pRSV-β-gal as an internal control. For GAL4 fusion experiments, 5 μg of GAL4-drosE2F1 expression vectors was cotransfected with 5 μg of pG5ElBCAT (27) as a reporter plasmid and 10 μg of pRSV-β-gal as an internal control. For the *Drosophila* polymerase α promoter experiment, 500 ng of pdPolαCAT was cotransfected with increasing amounts of pdrosE2F1$_{wt}$ (100 ng, 500 ng, and 2.5 μg) and 10 μg of pRSV-β-gal. Cells were harvested after 48 h and assayed for CAT and β-galactosidase activities as described previously (9). CAT activities were expressed relative to those of control vector plasmids. All experiments were done at least three times, and representative data are presented.

Antibody production. A rabbit antiserum to drosE2F1 was raised by injecting a New Zealand White rabbit with affinity-purified GST-drosE2F1 fusion protein containing aa 153 to 768.

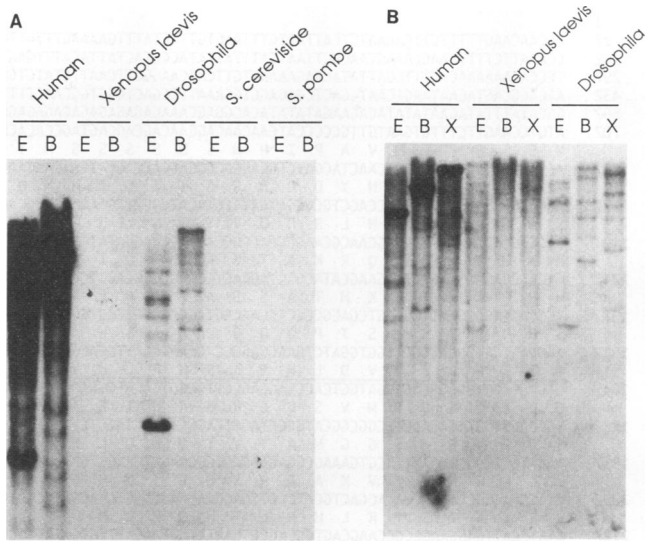

FIG. 1. Southern analysis of E2F1-hybridizing sequences in heterologous DNA. (A) DNAs isolated from human (20 μg), *X. laevis* (5 μg), *D. melanogaster* (5 μg), *S. cerevisiae* (5 μg), and *Schizosaccharomyces pombe* (5 μg) cells were digested with *Eco*RI (E) and *Bam*HI (B); Southern blots were prepared and probed with the 5′ half of the E2F1 cDNA as described in Materials and Methods. (B) DNAs isolated from human (20 μg), *X. laevis* (20 μg), and *D. melanogaster* (5 μg) cells were digested with *Eco*RI (E), *Bam*HI (B), and *Xba*I (X) and probed with the 3′ half of the E2F1 cDNA at reduced stringency as described in Materials and Methods.

RESULTS

E2F1-homologous sequences in other organisms. To identify potential sources for the isolation of an E2F1-equivalent gene, Southern blots containing digested DNA from *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Xenopus laevis*, and *D. melanogaster* were probed with a labeled fragment from the E2F1 cDNA that includes sequences that specify the DNA binding activity of the protein (9, 18, 24, 34). As shown in Fig. 1, although there was little or no specific hybridization to the yeast DNA samples, there was a clear signal with both the *Xenopus* and the *Drosophila* DNA samples (Fig. 1A). An E2F1 probe that encompassed sequence encoding the C-terminal activation and Rb binding domain also generated a clear signal with the *Drosophila* DNA sample (Fig. 1B). Given the indication of homology with *Drosophila* sequences, we proceeded to screen a *Drosophila* cDNA library.

Isolation of a *Drosophila* E2F cDNA clone. A λgt10 *Drosophila* library, prepared from poly(A)+ RNA from imaginal discs, was plated and probed with the same E2F1 sequences used for the Southern hybridizations. From the primary screen of 3×10^5 phage, 36 positives were picked and rescreened with the probe. A total of 23 clones continued to score as positive in the screening and were subjected to further analysis. Through an analysis of restriction enzyme digestion patterns, we were able to determine that the majority of the isolates represented the same sequence. The remainder of the initial positive clones fell into two additional groups, but these have not been analyzed further.

A representative cDNA clone that contained the largest insert was subjected to sequence analysis, the results of which are presented in Fig. 2. A single open reading frame extending from nt 702 to 3116 was identified, predicting a protein of 805 aa with a molecular mass of 87.5 kDa. A comparison of the *Drosophila* cDNA sequence with the human E2F1 sequence is shown in Fig. 3. Considerable homology can be found with sequences in the previously defined DNA binding domain of E2F1. In particular, there is as much as 69% identity of sequence within the helix-loop-helix motif of E2F1. An amphipathic appearance of hydrophobic amino acids is quite well conserved in regions corresponding to helix 1 and helix 2 of human E2F1, suggesting a putative helix-loop-helix structure in the corresponding region of the *Drosophila* product. There is also a considerable conservation of leucine-rich heptad repeats that overlap the putative loop domain and the C terminus of helix 2. In contrast, there was little homology with a stretch of basic residues that immediately precede the putative helix-loop-helix domain of E2F1, consistent with recent analyses that demonstrate a lack of importance of these residues in E2F1 function (9).

Although the most extensive homology is within this DNA binding domain of E2F1, there is additional sequence homology. Notably, the C-terminal region of the E2F1 sequence that includes the transcription activation domain exhibits approximately 28% homology to the C terminus of the *Drosophila* clone. Moreover, previous experiments have shown that the sequences involved in binding to the Rb protein are contained within this activation domain (18, 24). A comparison of these sequences with that of the *Drosophila* clone reveals an even greater homology, with approximately 50% identity of the amino acid residues.

A recent report has described the isolation of a distinct cDNA clone, termed DP-1, that also encodes a protein with properties of E2F (16). A comparison of the DP-1 sequence with that of the *Drosophila* cDNA clone shows little evidence for homology. At best, there is a 38% identity of residues in the

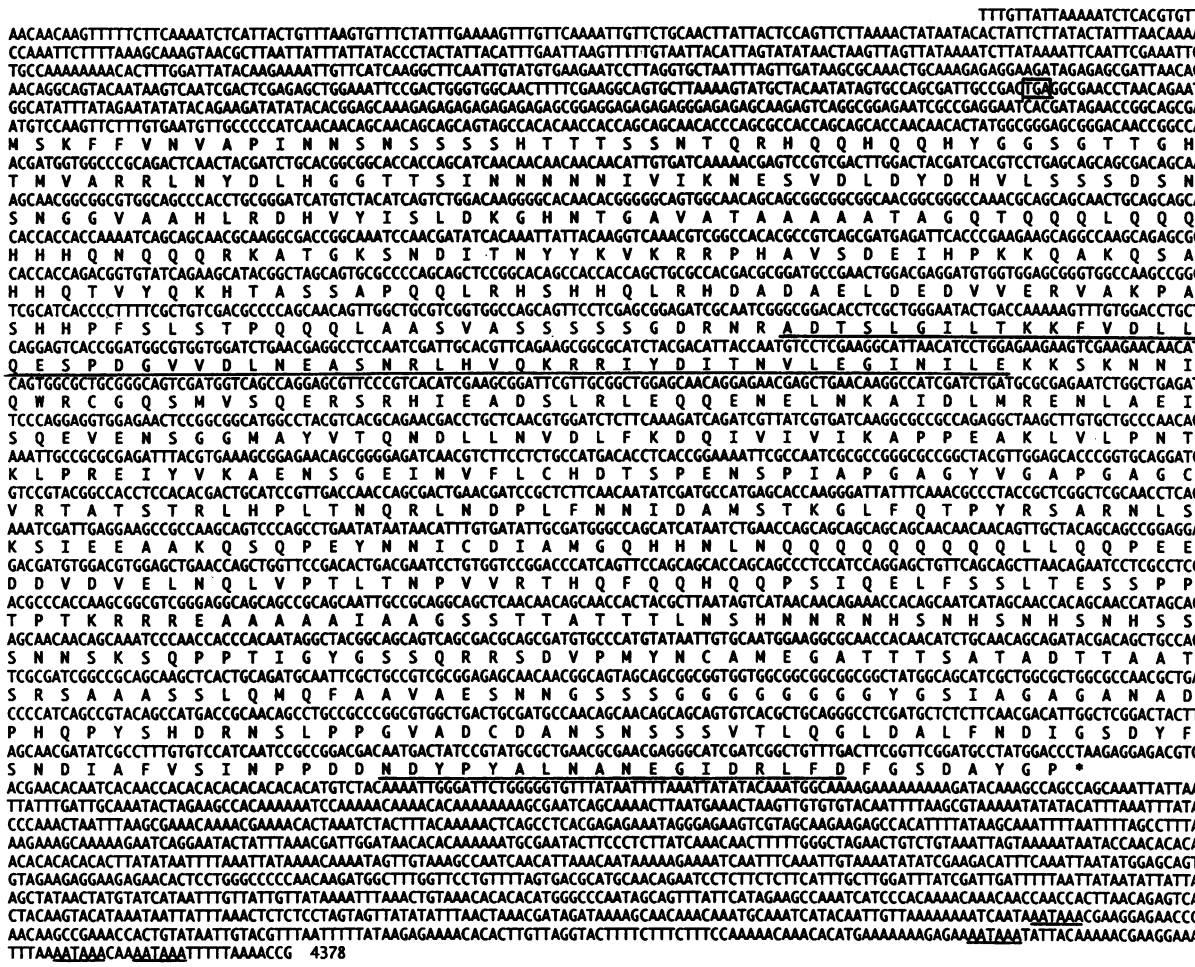

FIG. 2. Sequence of the drosE2F1 cDNA. Numbering of the nucleotide sequence is shown at the left; amino acid numbering is on the right. Amino acid sequences which have homologies to the helix-loop-helix motif of the DNA binding domain of E2F1 and to the Rb binding domain of E2F1 are underlined. A stop codon in the same reading frame in the 5′ untranslated region is boxed. Putative polyadenylation signals in the 3′ untranslated region are underlined.

region of the *Drosophila* cDNA sequence that has homology to the second helix domain of the human E2F1 gene. From these comparisons, it is clear that the *Drosophila* isolate is indeed related by sequence to the human E2F1 clone; on the basis of these extensive sequence similarities, we have termed this clone drosE2F1.

Using an approach similar to ours, Dyson et al. (14) have isolated a cDNA clone that encodes an identical protein. Nevertheless, sequence comparison reveals a divergence at position 353 relative to the sequence presented in Fig. 2, a result that we have also now observed in several independent cDNA isolates. The precise switch in sequence at this location raises the possibility that there is alternative splicing within these 5′ sequences.

The product of the drosE2F1 gene binds to an E2F recognition site. The extensive homology between the human E2F1 sequence and the *Drosophila* E2F1 sequence within the region important for DNA binding of E2F1 suggested the possibility that this *Drosophila* protein binds to DNA with E2F sequence specificity. Moreover, assays of *Drosophila* embryo extracts as well as *Drosophila* Schneider tissue culture cell extracts revealed a DNA binding activity with E2F sequence specificity (data not shown). In light of these considerations, we have assayed for the ability of the drosE2F1 product to bind to DNA.

The drosE2F1 cDNA insert was subcloned in a GST expression vector to allow expression of the drosE2F1 product as a fusion protein in *E. coli*. Following expression, the protein was purified by glutathione affinity chromatography and then analyzed for DNA binding capacity. As shown in Fig. 4, the drosE2F1-GST protein could indeed bind to the E2F-specific probe, as measured in a gel retardation assay. Specificity in binding was demonstrated by competition assays using a wild-type competitor or a mutant competitor in which the E2F recognition site is disrupted. Clearly, the drosE2F1 product binds to DNA, dependent on the E2F recognition sequence.

The drosE2F1 product activates transcription in *Drosophila* cells. Previous experiments have demonstrated the capacity of the E2F transcription factor to stimulate transcription, dependent on the interaction with the E2F recognition sequence (40). Recent studies have shown that the E2F1 product can activate transcription in a transfection assay, dependent on E2F1 sequences involved in DNA binding as well as transcriptional activation and dependent on the presence of E2F sites in the target promoter (9, 18, 34). Using a similar approach, we have assayed for the capacity of the drosE2F1 product to

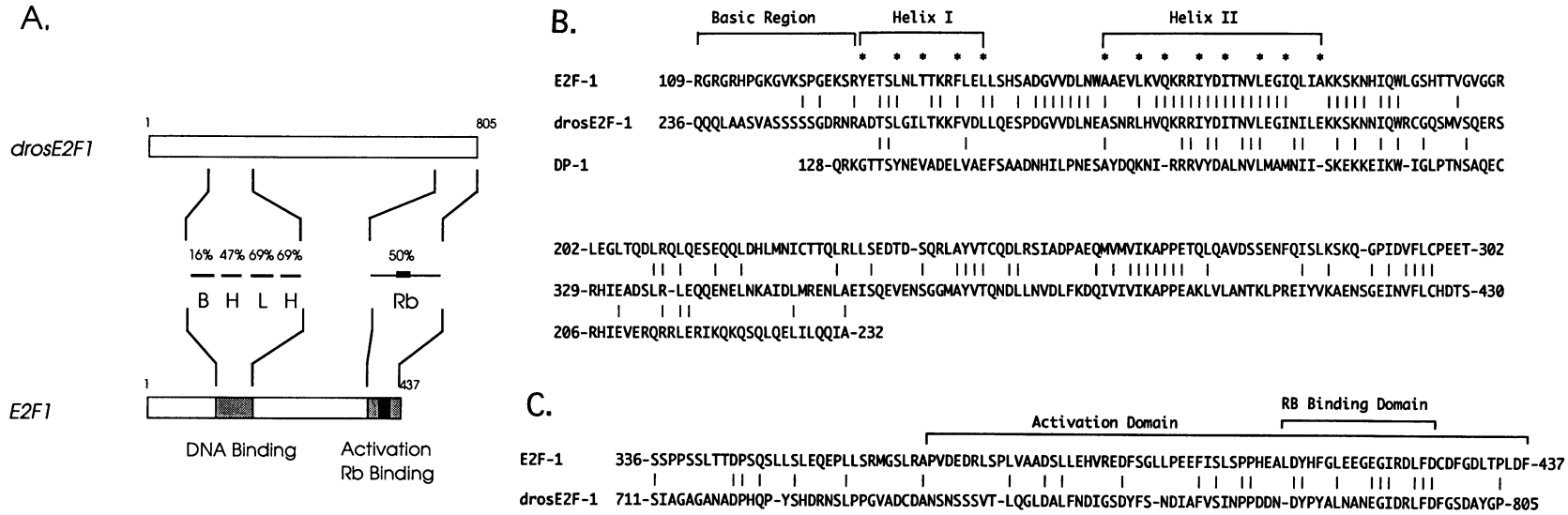

FIG. 3. Amino acid sequence homologies of drosE2F1 with human E2F1. (A) Schematic representation of homologous regions between drosE2F1 and human E2F1. The DNA binding domain of E2F1, including the basic-helix-loop-helix (BHLH) motifs, the transcription activation domain, and the Rb binding domain that is found within the activation domain, are indicated. (B) Regions of sequence homologies between drosE2F1, human E2F1, and murine DP-1. Amino acid sequences were aligned to maximize the homologies. Identical amino acids are indicated by a line. The basic region and helix-loop-helix region of human E2F1 are indicated. Asterisks indicate hydrophobic amino acid residues thought to be important for human E2F1 as amphipathic helices. (C) Homology of C-terminal regions between drosE2F1 and human E2F1. The transcriptional activation domain and Rb binding domain of human E2F1 are indicated.

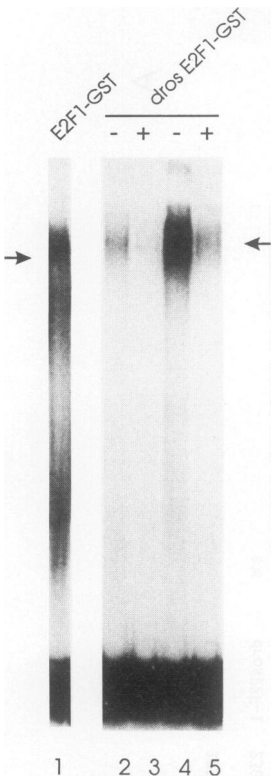

FIG. 4. DNA binding capacity of a GST-drosE2F1 fusion protein. Either 250 ng (lanes 2 and 3) or 1 μg (lanes 4 and 5) of a GST-drosE2F1 fusion protein was incubated with the wild-type E2F probe and assayed for binding. A 100-fold excess of mutant (lanes 2 and 4) and wild-type (lanes 3 and 5) competitor oligonucleotides was added as indicated. As a positive control, 300 ng of GST-E2F1 was assayed for binding to the same probe (lane 1).

activate transcription. Initial assays measured the capacity of the drosE2F1 product to activate E2F-dependent transcription upon transfection into the T98G human glioblastoma cell line. As shown in Fig. 5A, the human E2F1 product readily activated transcription, yielding an approximate 13-fold stimulation. In contrast, we observed no stimulation of transcription upon transfection of the drosE2F1 construct.

The failure of the drosE2F1 construct to activate transcription in T98G cells could be due to several factors, including species specificity. We thus used a *Drosophila* cell line to assay the activation potential of the drosE2F1 product. For these assays, we recloned the drosE2F1 insert into an expression plasmid under the control of the *Drosophila* actin promoter. This construct was then assayed for transcription activation potential by transfection into the *Drosophila* Schneider cell line together with a reporter plasmid consisting of the CAT gene under the control of a promoter with eight E2F recognition sites. As shown in Fig. 5B, CAT activity was stimulated more than 20-fold upon cotransfection of the drosE2F1-expressing plasmid. Mutation of the E2F sites in the CAT reporter plasmid abolished activation by the drosE2F1 product. We also assayed for the ability of the drosE2F1 product to stimulate transcription of a CAT gene under the control of the native adenovirus E2 promoter. As shown in Fig. 5C, the drosE2F1 product also stimulated expression from this promoter, and once again the activation was dependent on the integrity of the E2F recognition sites in the promoter. From these results, we conclude that the drosE2F1 clone encodes a protein with sequence as well as functional homology to the human E2F1 product.

Transcriptional activation function can be found in C-terminal sequences of drosE2F1. We have used two procedures in an attempt to identify amino acid sequences within the *Drosophila* E2F1 cDNA that contribute to transcriptional activation. First, stop codons that truncated the protein at aa

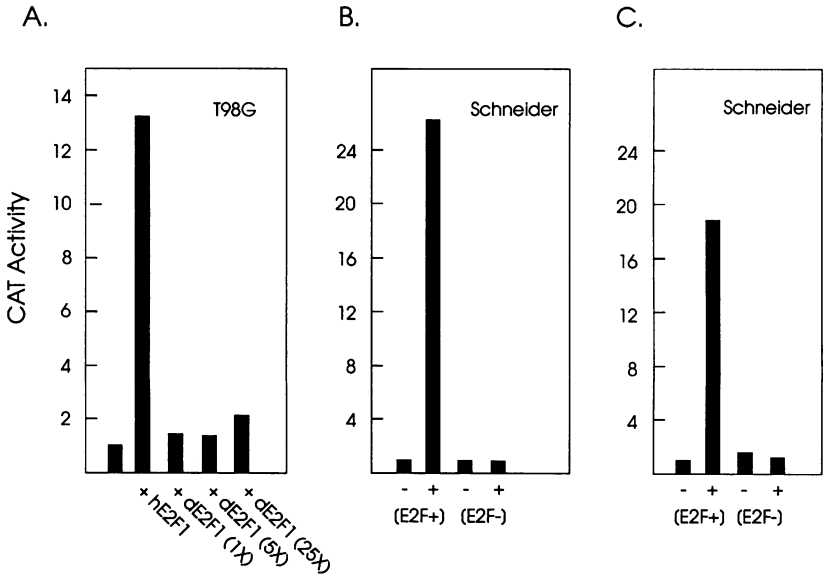

FIG. 5. The drosE2F1 product activates transcription in *Drosophila* cells. (A) Increasing amounts (100 ng, 500 ng, and 2.5 μg) of pCMV-drosE2F1 and 100 ng of pCMV-E2F1 were cotransfected into T98G cells with 5 μg of pE2WTx4CAT and 2 μg of pRSV-β-gal. Forty eight hours after transfection, cell extracts were prepared and assayed for CAT activity normalized for β-galactosidase activity. (B and C) pPac (−) and pdrosE2F1wt (+) (100 ng of each) were cotransfected into *Drosophila* Schneider cells with 5 μg of pE2WTx4CAT (E2F+) and pE2MT×4CAT (E2F−) (B) or pE2WTCAT (E2F+) and pE2CAT(E2F−) (E2F−) (C) and 10 μg of pRSV-β-gal. Relative CAT activities were obtained as for panel A.

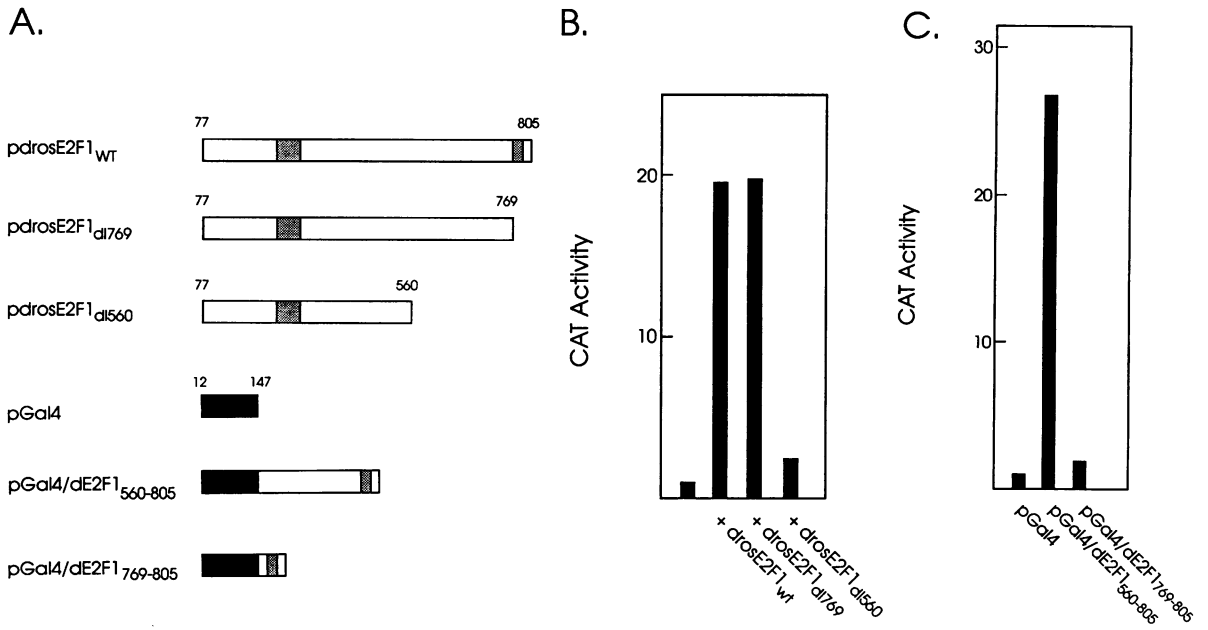

FIG. 6. Identification of a transcriptional activating domain of drosE2F1. (A) Schematic representation of the drosE2F1 and GAL4-drosE2F1 deletion mutants. The domains of drosE2F1 that are homologous to the E2F1 DNA binding and Rb binding domains are shaded. The GAL4 DNA binding domain (aa 12 to 147) is indicated by the black box. (B) pdrosE2F1$_{WT}$ (100 ng) and the deletion mutants pdrosE2F1$_{dl769}$ and pdrosE2F1$_{dl560}$ were cotransfected into Schneider cells with 5 μg of pE2WTx4CAT and 10 μg of RSV-β-gal. Relative CAT activities were obtained as indicated in the legend to Fig. 5. (C) pGal4 (5 μg) and the indicated GAL4-drosE2F1 expression vectors were cotransfected into Schneider cells with 5 μg of pE2WTx4CAT and 10 μg of pRSV-β-gal. Relative CAT activities were obtained as described in the legend to Fig. 5.

769 or 560 were introduced into the drosE2F1 cDNA (Fig. 6A). These constructs were then assayed for transactivation function. As shown in Fig. 6B, whereas removal of the C-terminal 36 aa (drosE2F1$_{dl769}$) resulted in little or no reduction of transcriptional activation, the removal of an additional 209 aa (drosE2F1$_{dl560}$) essentially eliminated transcriptional activation capacity.

A second assay used the yeast GAL4 DNA binding domain to generate chimeric proteins that included portions of the drosE2F1 C-terminal sequence (Fig. 6A). As shown in Fig. 6C, transcriptional activation function could be found in the C-terminal 245 aa of drosE2F1. In contrast, a GAL4 fusion containing the C-terminal 36 aa was essentially inactive. From these results, we conclude that a transcriptional activation domain can be identified between aa 560 and 769 within the C-terminal drosE2F1 product.

A potential gene target for the drosE2F1 product. The understanding of the role of E2F in mammalian cells has been guided by the realization that the transcription factor very likely plays a role in the activation of a group of genes that encode S-phase products. Thus, the promoters of genes such as the DHFR, thymidine kinase, DNA polymerase α, and ribonucleotide reductase genes all contain E2F recognition sites within critical promoter regions (30). Moreover, experiments of Slansky et al. have directly shown that the E2F sites within the DHFR promoter are critical for activation of transcription in late G_1 just prior to entry into S phase (36). If the drosE2F1 product bears functional homology to the mammalian E2F1 product, then we might expect that a similar group of genes would be targets for its action.

To identify potential *Drosophila* target genes for the drosE2F1 product, we have searched the gene sequence libraries to identify promoter sequences of the group of S-phase-specific genes suspected to be regulated by E2F in mammalian cells. Unfortunately, in only one such case has promoter sequences been identified. The promoter of the DNA polymerase α gene was recently identified and sequenced (21). Within the 5′ flanking sequence is an overlapping pair of E2F recognition sequences located at positions −353 to −342 relative to the transcription initiation site (Fig. 7A). In addition, there are at least two other sequences that bear a close resemblance to an E2F site, located at positions −21 to −14 and −12 to −5.

Using the *Drosophila* DNA polymerase α promoter as a probe, we have assayed for the presence of a drosE2F1-related binding activity in extracts from *Drosophila* Schneider cells. As shown in Fig. 7B, the overlapping sequence at −353 to −342 was indeed an effective binding site for an activity from Schneider cells. The E2F specificity is evident in the competition with the wild-type and mutant E2F site from the adenovirus E2 promoter (lanes 7 and 8). Binding to DNA polymerase α sites in addition to site 1 could also be detected, particularly site 2, but these sites were clearly less efficient than the −353 to −342 site.

Evidence that the binding activity detected in the Schneider cell extract reflects binding of the drosE2F1 product is indicated by the results shown in lane 10 of Fig. 7B, in which a rabbit antiserum raised against the GST-drosE2F1 protein was able to eliminate the binding complex. In contrast, the preimmune serum had no effect on the binding activity. From these results, we conclude that the DNA polymerase α promoter does indeed contain E2F recognition sites, at least one of which is of high affinity.

In light of the observation that the drosE2F1 protein recognizes and binds to multiple sites within the DNA polymerase α promoter, we have tested whether this promoter can be activated by the drosE2F1 product in a transient transfection assay. As shown in Fig. 8, a CAT gene under the control of the

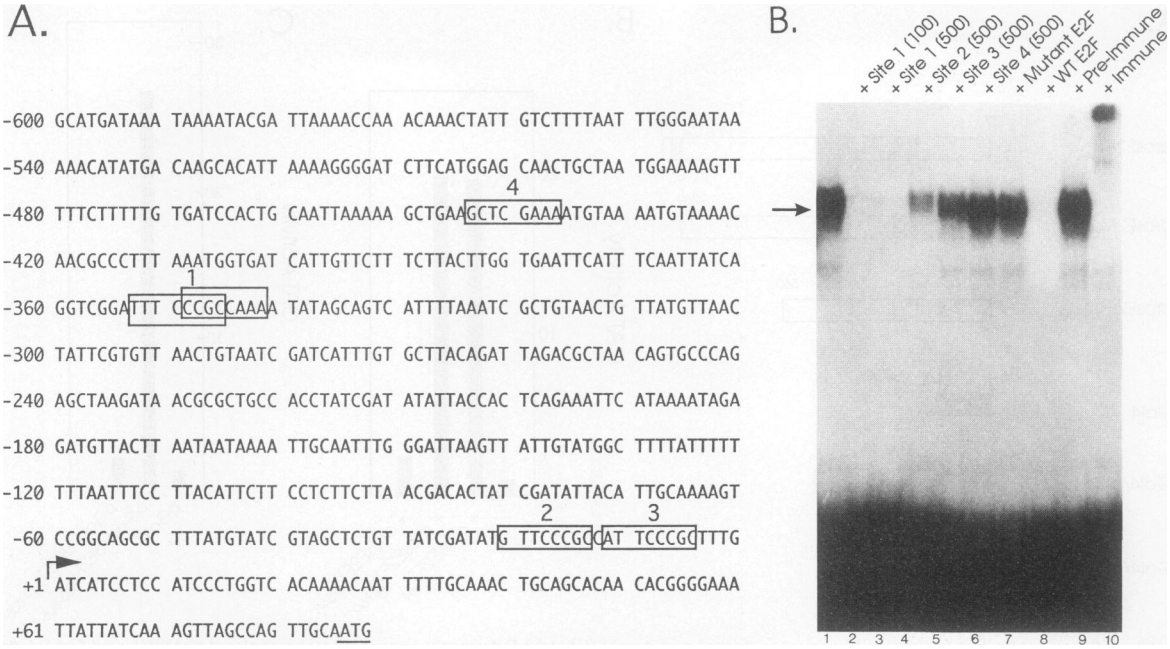

FIG. 7. The *Drosophila* DNA polymerase α promoter contains E2F binding sites. (A) Potential E2F recognition sites in the *Drosophila* DNA polymerase α promoter. The sequence of the promoter as determined by Hirose et al. (21) is presented, with the transcription initiation site numbered as +1 and indicated by an arrow. The ATG initiation codon is underlined. The potential E2F recognition sites are boxed and numbered 1 to 4. (B) E2F DNA binding activity in Schneider cell extracts. *Drosophila* Schneider cell nuclear extract (7 μg) was incubated with a DNA probe representing site 1 and then assayed for binding by gel retardation. Competitions were performed with the indicated unlabeled DNAs (amount indicated in molar excess) as well as mutant and wild-type (WT) E2F site DNA from the adenovirus E2 promoter. Finally, the binding assay using the site 1 probe was incubated in the presence of preimmune rabbit serum and immune serum raised against the GST-drosE2F1 protein. Each serum was added at a 1:320 dilution.

DNA polymerase α promoter was stimulated up to 10-fold by cotransfection of the drosE2F1 cDNA. In contrast, the control Rous sarcoma virus promoter was unaffected by cotransfection of the drosE2F1 product.

Although the transfection results clearly demonstrate that the drosE2F1 product can activate the DNA polymerase α promoter, two observations must delay our conclusion that this gene is directly activated by the drosE2F1 product. First, it is apparent in the assay of Fig. 8 that the drosE2F1-mediated activation of the DNA polymerase α promoter is considerably less than that of the test promoter that contains eight E2F binding sites. Second, initial attempts to eliminate activation by the drosE2F1 product through mutation of the high-affinity binding site have not succeeded. Moreover, previous analyses of the *Drosophila* DNA polymerase α promoter have found that deletion of sequence upstream of −233 does not impair promoter activity (21). Of course, activation could be mediated by the two additional sites that are within the region defined as critical for transcription. It also remains possible that the relative importance of the high-affinity site depends on the circumstances of cell growth. Clearly, the critical next experiment must be an in vivo determination, in adult or developing flies, of whether the DNA polymerase α gene is activated by the drosE2F1 product.

DISCUSSION

A variety of observations suggest that the human E2F transcription factor is likely to be evolutionarily conserved. Although originally identified as a transcription factor activity that was critical for the expression of the adenovirus E2 gene in response to the viral E1A product (25), more recent experiments have demonstrated a role for E2F in cell cycle-related transcription control. In particular, at least a part of the growth-suppressing activity of Rb appears to involve control of the E2F transcription factor. Moreover, recent experiments have shown that the Rb-mediated arrest of cell growth in G_1 can be overcome by the expression of the E2F1 product (41). Furthermore, other experiments have shown that overexpression of the E2F1 product in an otherwise quiescent cell can stimulate such cells to enter S phase in the absence of other growth signals (23).

The transcriptional activation of a group of genes that encode products involved in DNA synthesis and that are coregulated at the G_1/S phase transition appears to be directed by E2F (30). The products of these genes, such as DHFR, thymidine kinase, and DNA polymerase α, are highly conserved during evolution, and thus one might anticipate that the regulatory system controlling the expression of these genes would also be conserved. The results presented here describe the isolation of a *Drosophila* clone with properties of the human E2F1 gene.

It is becoming increasingly evident from recent work that multiple gene products likely contribute to what is defined as E2F. Although the initial E2F1 clone has now been isolated multiple times (18, 24, 34), an additional cDNA, termed DP-1, that exhibits properties of E2F but that is distinct from the E2F1 clone has been isolated (16). Recent evidence demonstrates that the E2F1 product and the DP-1 product can physically interact to generate a heterodimeric factor that may be the natural form of E2F (3, 19). Moreover, through screening of human cDNA libraries with the original E2F1 clone, additional related cDNAs have now been isolated (22a,

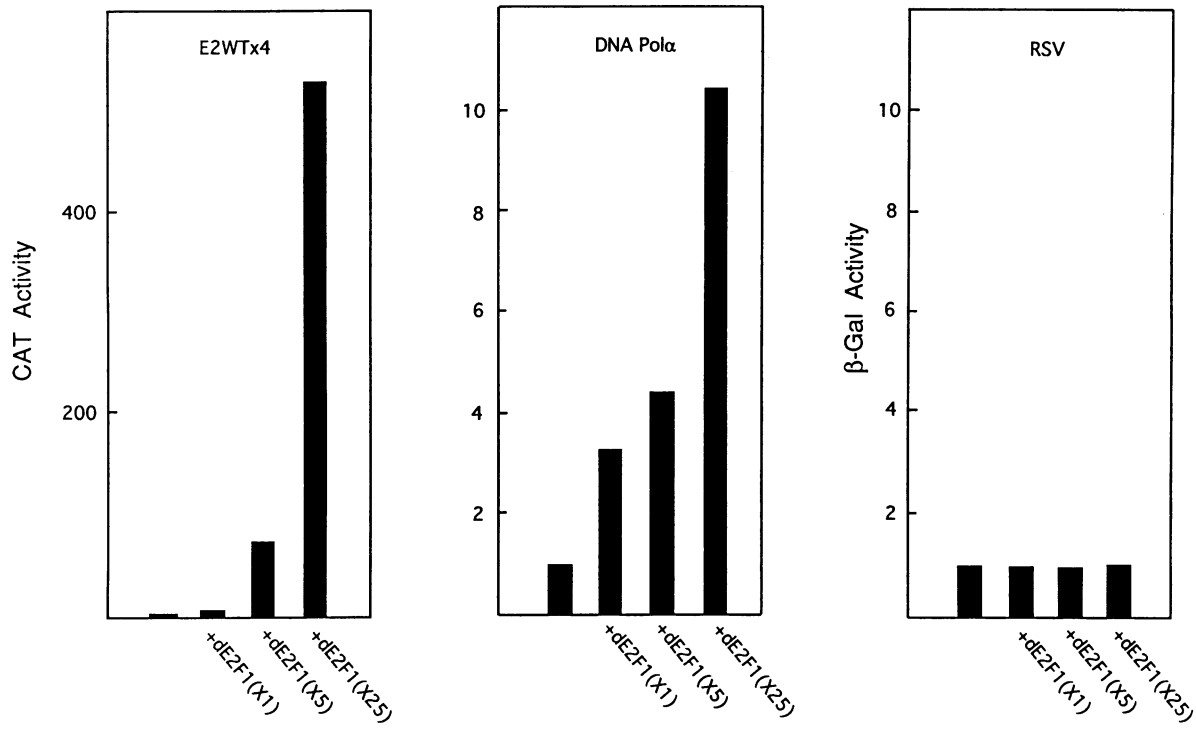

FIG. 8. The drosE2F1 product activates transcription directed by the DNA polymerase α promoter. Schneider cells were transfected with either pE2WTx4CAT (2.5 μg) or pdPolαCAT (0.5 μg) together with increasing concentrations of pdrosE2F1wt (1× = 100 ng) and a constant amount of pRSV-β-Gal. Relative CAT activities were obtained as indicated in the legend to Fig. 5. For comparison, the β-galactosidase (β-Gal) activities are presented separately in the rightmost panel. Polα, polymerase α; RSV, Rous sarcoma virus.

26a). At this stage, the relationship of these various clones to one another or to the various functional properties of E2F is not yet clear. One possibility is that distinct E2F activities, as defined as products of the various cDNA clones, are involved in separate activities of E2F such as binding to Rb and binding to p107. Clearly, the involvement of multiple gene products in the function of E2F complicates the functional analyses. One approach to sorting out these events is through the analysis of specific mutations in individual genes that encode the various E2F activities. The isolation of a *Drosophila* gene that may be a counterpart of the mammalian E2F1 affords the possibility of such an approach.

A significant factor in the recent progress in understanding mammalian E2F function has been the identification of potential target genes that might be subject to E2F control. These include genes activated at the G_1/S transition that encode activities playing a role in S-phase events (30). In considering the possibility that a similar control pathway may be found in *D. melanogaster* as well, our search for evidence of E2F recognition sites within promoters of equivalent *Drosophila* genes led us to the DNA polymerase α gene and the finding that the drosE2F1 product may indeed regulate the activity of this gene. Of course, these results indicate only that the drosE2F1 product can regulate the DNA polymerase α promoter; we must await further experiments to determine whether it actually does so within the developing or adult fly. Nevertheless, the presence of functional E2F binding sites within this gene that is likely regulated by E2F in a mammalian cell strongly suggest that this will be the case. Unfortunately, promoter sequence information is not available for additional members of the family of S-phase genes. Nevertheless, we anticipate that, as with the studies of mammalian E2F, an identification of the targets for drosE2F1 action, coupled with analyses directed at the interactions involving the drosE2F1 product as well as the effects of altered expression of the drosE2F1 product, will be an important approach to developing a better understanding of the role of E2F in cellular function.

Finally, although the analyses of the drosE2F1 gene strongly suggest a relationship to the mammalian E2F1 gene, important questions remain to be addressed. One of the most critical concerns the existence of analogous growth-regulatory proteins that control E2F activity in a mammalian cell. To date, there has been no description of a *Drosophila* equivalent of Rb or the Rb-related p107 protein, both of which interact with and regulate E2F activity in mammalian cells. Nevertheless, it is a reasonable assumption that at least the S-phase-specific events involving E2F, including the activation of S-phase genes as well as the interaction with the cyclin A-cdk2 kinase complex, will likely be evolutionarily conserved. In this context, the 50% homology between the C terminus of the drosE2F1 product and the Rb binding domain of the human E2F1 product is striking, suggesting the presence of a drosE2F1-interacting molecule in *Drosophila* like the mammalian activities that interact with E2F1.

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