DNA synthesis, such as E2F, governs the transcription of a broad panel of genes whose most promoters that have been analyzed to date, E2F is a heterodimer composed of an E2F polypeptide and a DP peptide (DeGregori et al. 1995; Dyson 1998). Activation of cyclin D- and cyclin E-dependent kinases leads to the activation of E2F, thus allowing the transcriptional program to be tightly coupled with cell-cycle position. Multiple lines of evidence show that E2F is rate limiting for S-phase entry. Overexpression of E2F genes is sufficient to drive cells into S phase; in contrast, the inhibition of E2F activity reduces S-phase entry. The consequences of ectopic E2F activity vary greatly in different cell types. In some cells the overexpression of E2F genes leads to cell proliferation; in others, E2F activity induces apoptosis (Wu and Levine 1994; Singh et al. 1994).

E2F is a heterodimer composed of an E2F polypeptide and a DP peptide (Girling et al. 1993; Wu et al. 1995). Currently, six E2F genes and two DP genes have been found in mammalian cells. In general E2F has been viewed as a transcriptional activator that promotes cell-cycle progression. However, several studies have shown that E2F can function as a transcriptional corepressor when bound to the RB tumor suppressor protein (pRB; Hamel et al. 1992; Weintraub et al. 1992, 1995). In most promoters that have been analyzed to date, E2F binding sites appear to confer cell-cycle regulation by mediating cell-cycle repression of the promoter in G0 or G1 phase. The DHFR promoter provides the only well-characterized example, in which cell-cycle regulation is due to E2F-mediated activation. The molecular mechanisms that allow E2F complexes to activate or repress transcription are not well understood. However, recent studies showing that pRB binds to histone deacetylases.
have raised the possibility that pRB represses transcription by altering chromatin structure (Breitm et al. 1998; Luo et al. 1998; Magnaghi-Jaulin et al. 1998). Broad inhibitors of histone deacetylases eliminate pRB's ability to repress several different transcription factors. Conversely, transcription activation by E2F-1 is potentiated by the CBP histone acetylase (Trouche and Kouzarides 1996).

We and others have isolated Drosophila genes encoding homologues of E2F and DP proteins (Dyonlacht et al. 1994; Ohtani and Nevins 1994; Hao et al. 1995). Throughout this article the dE2F/dDP heterodimers are referred to as E2F and the individual proteins as dE2F and dDP. dE2F null embryos lack a G1-S transcriptional program as assayed by the expression of the ribonucleotide reductase small subunit (RNR2) and proliferating cell nuclear antigen (PCNA) genes (Duronio et al. 1995). dE2F mutants still support a reduced rate of DNA synthesis compared to wild-type embryos (Duronio et al. 1995, 1998; Royzman et al. 1997). Consistent with this, the level of cyclin E mRNA is reduced but not eliminated in dE2F mutants (Royzman et al. 1997; Duronio et al. 1998). dE2F mutant animals die later in development at the late larval/pupal stage. In dE2F mutants, larval growth is slowed compared to wild-type animals and some animals develop melanotic pseudotumors (Royzman et al. 1997). The dDP null animals also die during the late larval/pupal stages and have a mutant phenotype that is similar to, but less severe than, the dE2F nulls (Royzman et al. 1997; Duronio et al. 1998).

In vivo studies suggest that E2F cannot be viewed simply as a positive regulator of cell proliferation. E2F-1 knockout mice have a tumor phenotype and defects in apoptosis and tissue morphogenesis (Field et al. 1993; Yamasaki et al. 1996). These results, together with analysis of animals carrying both Rb and E2F1 mutant alleles, indicate that the loss of E2F1 activity can lead either to tumor development or to reduced cell proliferation depending on the cell type (Pan et al. 1998; Tsai et al. 1998; Yamasaki et al. 1998). E2F-5 knockout mice develop hydrocephalus, caused by the excessive secretion of cerebrospinal fluid, but have no overt defects in cell proliferation. In Drosophila, the analysis of dE2F mutant clones suggests that dE2F has functions in postmitotic cells. Taken together, these experiments suggest that E2F proteins have functions that are essential for cell proliferation, differentiation, and apoptosis.

The Drosophila compound eye provides an excellent system for studying E2F because the coordination of cell proliferation, differentiation, and apoptosis have been characterized in great detail during eye development (Wolff and Ready 1993). The compound eye develops from a columnar sheet of epithelium called the eye imaginal disk. During the third larval instar a wave of differentiation, called the morphogenetic furrow, passes from posterior to anterior across the eye disk. Cells anterior to the furrow are undifferentiated and divide asynchronously. As cells enter the furrow they become synchronously arrested in G1. Upon leaving the furrow, groups of cells differentiate into specific photoreceptor cells. The remaining cells do not differentiate immediately posterior to the furrow but instead undergo another round of S phases called the second mitotic wave. After this second division, these cells differentiate into the remaining photoreceptor cells and the cone, bristle, and pigment cells.

Both dE2F and dDP are expressed in the eye imaginal disk. dE2F is expressed in a thin stripe anterior to the morphogenetic furrow where cells are entering mitosis and in a broad region posterior to the furrow where cells have exited mitosis and are differentiating into neuronal cells (Asano et al. 1996; Brook et al. 1996; Du et al. 1996b). Overexpression experiments and clonal analysis reveal multiple aspects of E2F function in the eye. The coexpression of dE2F and dDP posterior to the furrow using the glass-responsive GMR promoter (Ellis et al. 1993) causes ectopic S phases in cells that are normally postmitotic (Asano et al. 1996; Du et al. 1996b). Most S phases are seen in uncommitted cells, although some cells that initiate photoreceptor differentiation can also be driven into the cell cycle. This increase in S phases is largely counterbalanced by an increase in apoptosis (Asano et al. 1996; Du et al. 1996b). When dE2F/dDP-induced apoptosis is suppressed by coexpression of the baculovirus cell death inhibitor protein p35, the number of cells seen between the ommatidial clusters increases dramatically (Du et al. 1996b). dE2F mutant clones in the eye disk are difficult to recover and are considerably smaller than the accompanying wild-type twin spot, suggesting that dE2F is required for either cell proliferation or cell viability (Brook et al. 1996b). In addition, dE2F mutant clones have variable numbers of photoreceptors and some rhabdomeres display an abnormal morphology, suggesting a role for dE2F in these postmitotic cells.

Flies carrying P[w+; GMRdE2F] and P[w+; GMRdDP] transgenes have been described previously (GMR-dE2FdDP) and have eyes that appear rough (Du et al. 1996b). This phenotype is sensitive to the number of transgenes and is modified by mutations in genes that have been shown to affect E2F activity. In particular the GMR-dE2FdDP phenotype is suppressed by coexpression of the Drosophila RB homologue, RBF (Du et al. 1996a), and it is enhanced by loss-of-function mutations in the rbf gene (Du and Dyson 1999). In this article we describe the results of several genetic screens to isolate novel regulators of E2F activity through the isolation of mutations that enhance or suppress the GMR-dE2FdDPp35 phenotype. Using this strategy we recovered mutations in six different genes that enhance the phenotype. The enhancer loci that we isolated from these screens include brmA (brm), moir (mor), and osa, three members of the trithorax group of genes, pointd (pnt), an ETS family transcription factor, and...
polyccephalon (poc), a regulator of Deformed homeotic function. Mutations in poc also suppress the eye phenotype of GM R-p21 animals, indicating a function for this protein in several cell-cycle pathways.

**MATERIALS AND METHODS**

**Drosophila stocks:** Drosophila were cultured on standard cornmeal/molasses/yeast media at 25°C. The poc alleles were generated in the McGinnis lab and are described in Gellon et al. (1997). The E(Raf)2A alleles were a gift of Barry Dickson and are described in Dickson et al. (1996). GM R-p21 and GM R-dacapo flies were a gift of H. Hariharan and the GMR-p35 flies were from Bruce Hay. All of the other fly stocks used in our research were obtained from the Bloomington, Indiana, stock center. Descriptions and references for all of the alleles described in this article can be found in Lindsey and Zimm (1992) or through the Flybase computer database (http://flybase.harvard.edu:7081).

**GMR-E2F, DP, p35 genetic interaction tests:** Alleles of the following cell-cycle genes were tested for their ability to modify both the GM R-dE2FDP and GM R-dE2FDPp35 phenotypes: vrl161 (dDP point mutant), vg16 (dDP deficiency), E2F296, E2F2127, dcd12, dcd21, dcd218, Df(2R)H81 (dcd2 deficiency), cylinda1214, Df(3R)Z94A (b cyclin B deficiency), cylinda6197, cylindaE19, stg16, and stg18.

The following mutations in members of the epidermal growth factor (EGF) and sevenless pathways were tested for a genetic interaction with the GM R-dE2FDP-driven phenotypes: E(sav)3C106 (Ras1 hypomorph), E(sav)3C40 (Ras1 hypomorph), Gap11410, Gap116, yan1 (yan allele), Egr2 (Ellipse), Egr3, sev16, raf112, raf1112, and raf16.

Alleles of the following trithorax group and Polycomb group genes were tested for their ability to modify the GM R-dE2FDPp35 phenotype: Dll12, ecpi, poc1, Pchl, trx1, In(2R)Pcl11, Scm13, scx1, Pchl, Dll3, Dll5, kbt2, trx2, dev12020, and kis1.

**Scanning electron microscopy:** Adult flies were dehydrated in a series of ethanol washes and examined by scanning electron microscopy using the procedures from Kimmel et al. (1990).

**Mutagenesis screen:** w1185; is02; is03 males for EMS mutagenesis (gift of I. Hariharan) were starved for 2 hr and then fed a 1% sugar solution containing 25 mm EMS (Sigma, St. Louis) for 14–16 hr. For X-ray mutagenesis, 0- to 2-day-old isogenic males were aged for 2 days and then were given a 4000-rad dose (1 mA, 100 kV) of radiation. After mutagen treatment the males were crossed to homozygous GM R-dE2FDPp35 females and allowed to mate for 4 days. The eyes of the progeny were examined under a dissecting scope and male flies that showed enhancement of the phenotype were backcrossed to the GM R-dE2FDPp35 females. The mutations that retested were balanced and placed into complementation groups. The total number of F1 progeny examined was estimated from counting the number of male flies in a single bottle and then multiplying by the number of bottles used in the screen. Approximately 40,000 mutagenized chromosomes were screened with EMS and 4100 chromosomes were screened with X rays. The GM R-dE2FDPp35 flies were recombined by recombining a second chromosome GM R-p35 line onto a chromosome carrying one copy of GM R-dE2F and one copy of GM R-dDP.

One representative allele of each of the six complementation groups was meiotically mapped using linkage to the recessive male marker a1, dpl, b, pr, c, px, sp was used to map the second chromosome mutations and linkage to r, h, th, st, cu, sr, e, ca was used to map the third chromosome mutations. Once the chromosomal locations of the mutants were found using meiotic mapping, deficiencies and known mutations in the relevant regions were tested to determine if they failed to complement the enhancer mutations. poc2118, E(Raf)2A1061, E(Raf)2A1877, Df(2L)IPM, Df(2L)TE21A, and Df(2L)net-PM 47C failed to complement E(E2F)2A mutations. E(E2F)2A3A (brm) mutations were uncovered by Df(3L)brm11, Df(3L)ht102, and brm1, mor1, mor2, and mor5 failed to complement E(E2F)3B mutations. Df(3R)DG2 and osa1 uncovered E(E2F)3C mutations and pnt1072539 and pnt1072539e failed to complement E(E2F)3D mutations. Based on the meiotic mapping E(E2F)2B maps between markers dp (25A) and b (34D) at ±28F/29A.

**Brdu analysis of eye imaginal disks:** Isogenic cn bw sp and poc2118; 2(2R)GLa,BcElp males were mated to homozygous GM R-p21 females. Larvae heterozygous for poc2118 were identified by the absence of the Black cell (8c) larval marker. Eye disks from wandering third instar larvae were dissected in Schneider’s media and then incubated in BrdU (0.5 mg/ml in Schneider’s) for 30 min to 2 hr. The disks were washed in Schneider’s for 10 min, washed in PBS for 20 min, and then fixed for 30 min at room temperature in 4% formaldehyde in PEM buffer (100 mm PIPES pH 7, 2 mm EGTA, and 1 mm MgSO4). After fixation the disks were washed in PBS/0.3% Triton X-100 for 10 min, incubated in PBS/0.6% Triton X-100 for 30 min, and washed in methanol for 30 min. The disks were rehydrated through a methanol/PBS series and then transferred to PBS/0.3% Triton X-100 + 2N HCL for 30 min. The disks were washed twice in PBS for 10 min and then were blocked for 30 min in PBS/0.3% Triton X-100/10% normal goat serum (NGS). After the blocking step, the eye disks were incubated overnight at 4°C in a 1:100 dilution of mouse anti-BrdU (Becton Dickinson, San Jose, CA) in PBS/0.3% Triton X-100/10% NGS. The disks were then washed in PBS/0.3% Triton X-100 and the blocking step was repeated. Secondary antibody incubations were done at room temperature for 2 hr using a 1:100 dilution of horseradish peroxidase-conjugated goat anti-mouse (Bio-Rad, Richmond, CA) in PBS/0.3% Triton X-100/10% NGS. The disks were then washed in PBS and developed using DAB (diaminobenzidine).

**In situ hybridizations:** RNR2 digoxigenin riboprobes were synthesized using the Tautz’ kit (Boehringer Mannheim, Indianapolis) and in situ hybridizations were done according to the methods of Tautz and Pfeiffet (1989). The template plasmid for the riboprobe synthesis is described in Duronio and Farrel (1994).

**RESULTS**

**Mutagenesis screen:** Coexpression of dE2F and DDP in the eye imaginal disc using either the GMR promoter (Ellis et al. 1993) or the heatshock promoter causes a localized increase in the incorporation of BrdU as well as an increase in apoptotic cells (Asano et al. 1996; Du et al. 1996b). The eyes of GM R-dE2FDP flies have extrasensory bristles and the regular pattern of bristle, cone, and photoreceptor cells is disrupted, causing the eyes to appear rough. Several observations suggested that this phenotype might be suitable for a modifier screen. First, it is characterized by E2F-driven cell-cycle progression and apoptosis, two well-known properties of E2F. Second, the severity of the phenotype depends on the copy number of GMR-driven transgenes, suggesting that the phenotype is not saturated and that dominant modifiers might be isolated. Third, the phe-
notype is sensitive to changes in the level of RBF, the one known regulator of E2F in Drosophila. Deficiencies that uncover the rbf gene enhance the phenotype, whereas the overexpression of RBF from GM R-RBF transgenes suppresses the phenotype (Du et al. 1996a; Du and Dyson 1999).

We tested other loss-of-function mutations in cell-cycle genes for their ability to enhance or suppress the GM R-dE2FdDP phenotype. As the GMR promoter allows dE2F and dDP to be expressed 5- to 10-fold higher than the endogenous proteins, it was not surprising that the GM R-dE2FdDP phenotype was unaffected by mutations in dE2F and dDP. Surprisingly, however, this phenotype was also unaffected by mutations in cell-cycle genes such as cyclin A, cyclin B, cyclin E, cdc2, cdc2c, and string (data not shown). As RBF is thought to be regulated by cyclin-dependent kinases, this may suggest that the GM R-dE2FdDP phenotype is not sensitive to changes in signaling components that act upstream of RBF. Alternatively, the phosphorylation of RBF may not be rate limiting for the regulation of E2F activity in these flies.

A pilot screen was carried out (see materials and methods) to determine whether additional dominant enhancers and suppressors of the GM R-dE2FdDP phenotype could be isolated. A total of 4400 chromosomes were screened and four enhancer mutations were identified. The four mutations were crossed to other GMR-driven phenotypes [GM R-reaper (rpr), GM R-Rho1, GM R-p21, and GM R-dE2FdDPp35] to eliminate false positives that act by modulating transcription from the GMR promoter. One of the mutations enhanced every GMR phenotype tested; two mutations enhanced all the phenotypes tested except for GM R-p21; and the fourth mutation, E65, enhanced only the GMR-driven E2F phenotypes. The E65 mutation caused a subtle enhancement of the GM R-dE2FdDP phenotype but dramatically enhanced the phenotype with p35 present (Figure 1). The enhancement of the GM R-dE2FdDPp35 phenotype was striking and suggested that modifiers could be more readily found in a background in which E2F-induced apoptosis was no longer a major factor. We therefore carried out the screen for modifying mutations using the GM R-dE2FdDPp35. Enhancers and suppressors were tested against the GM R-dE2FdDP phenotype in a secondary screen, and against GM R-p35 to make sure the mutations were affecting E2F activity and not acting through p35.

We screened ~40,000 chromosomes that were mutagenized with EMS and 4100 chromosomes that were mutagenized with X rays (see materials and methods).
TABLE 1

Summary of GMR-dE2F,dDP,p35 enhancer complementation groups

<table>
<thead>
<tr>
<th>Complementation group</th>
<th>Location</th>
<th>Alleles</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>E(E2F)2B</td>
<td>25A-34D</td>
<td>pjc-10, pjc-1, ksh-5</td>
<td>Unknown</td>
</tr>
<tr>
<td>E(E2F)3A</td>
<td>72A3-5</td>
<td>pjc-2, ksh-6, ksh-7, ksh-8</td>
<td>brhma (brm)</td>
</tr>
<tr>
<td>E(E2F)3B</td>
<td>89A11-B4</td>
<td>ksh-9, ksh-10</td>
<td>moira (mor)</td>
</tr>
<tr>
<td>E(E2F)3C</td>
<td>90B1-D1</td>
<td>pjc-3, E65, ksh-11, ksh-12, ksh-13</td>
<td>osa</td>
</tr>
<tr>
<td>E(E2F)3D</td>
<td>94E</td>
<td>pjc-6, pjc-5, ksh-14</td>
<td>pointed (pnt)</td>
</tr>
</tbody>
</table>

a The map positions of brm, moira, osa, and pnt are from Lindsley and Zimm (1992). The map position of poc is described in Gellon et al. (1997). E(E2f)2B maps between the markers dp (25A) and b (34D).

b Indicates an X-ray allele.

c Indicates an X-ray allele.
ods). Thirty-eight enhancers and one suppressor of the GMR-dE2FdDPp35 phenotype were isolated. Enhancer stocks that were homozygous viable, that had a dominant rough eye phenotype, or that enhanced all the GMR-driven phenotypes tested were not considered further. Inter se crosses were done on the remaining 30 enhancers to determine the number of complementation groups. Twenty-four of the mutations sorted into six complementation groups; two groups were on the second chromosome and four on the third chromosome (Table 1). The six mutations that did not fall into one of the six groups may represent dominant mutations, multiple alleles of a viable locus, or loss-of-function mutations in lethal genes where only one allele was isolated (Table 2). The single suppressor isolated was homozygous viable and suppressed every GMR-driven phenotype tested, suggesting that it probably acts to regulate transcription from the GMR promoter. In parallel, we also screened the Bloomington Stock Center’s deficiency library collection for deletions that, when heterozygous, enhance the GMR-dE2FdDPp35 phenotype. This collection included deficiencies on the X, 2nd, and 3rd chromosomes and four deficiencies were recovered that enhance GMR-dE2FdDPp35 (see Table 2). Two of the deficiencies overlap in the 63A-63D region of the third chromosome, suggesting that a dosage-sensitive modifier of the E2F phenotype lies within that interval. Surprisingly, all of the deficiencies complemented mutations from each of the six complementation groups, suggesting that they remove different enhancer mutations and that the screen is not saturated. To date we have been unable to identify the genes that are removed by these deficiency intervals and responsible for the enhancement.

One representative allele from each complementation group was mapped to a chromosomal location by meiotic mapping (see materials and methods). Deiciencies and known mutations in the relevant regions were tested and the identities of five out of the six groups were determined (see Table 1). E(E2f)2A mutations are new alleles of polycephalon (poc). Three of the groups on the third chromosome are new mutations in pointed (pnt). The fourth group on the third chromosome contains mutations in pointed (pnt). The remaining locus on the second chromosome is novel and we have named it E(E2f)2B.

Characterization of identified loci: In the presence of the enhancers, the GMR-dE2FdDPp35 eyes were rougher and shinier and the arrangement of ommatidia was highly irregular (Figure 2). Patches with multiple bristles were seen as well as barren areas without bristles and discernible ommatidia. The enhanced eyes also had dark necrotic-like patches that increased in severity with age (data not shown). Sections of the enhanced eyes were uninformative due to the severity of the phenotype (data not shown). The morphology of the sections was grossly abnormal and we could not determine a specific defect in one particular cell type. Alleles from each of the six complementation groups were tested for their ability to enhance the GMR-dE2FDP phenotype without

TABLE 2

Summary of GMR-dE2F,dDP,p35 single enhancer mutations

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Chromosome</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>pjc-16g</td>
<td>2</td>
<td>58E-60C</td>
</tr>
<tr>
<td>ksh-15g</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>ksh-16g</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>ksh-17g</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>ksh-18g</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>ksh-19g</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Df(3L)M21</td>
<td>3</td>
<td>62F-63D</td>
</tr>
<tr>
<td>Df(3L)R370</td>
<td>3</td>
<td>63A1-64D10</td>
</tr>
<tr>
<td>Df(2R)PuD17</td>
<td>2</td>
<td>57B4-58B</td>
</tr>
<tr>
<td>Df(1)C128</td>
<td>1</td>
<td>7D1-6</td>
</tr>
</tbody>
</table>

a The map positions of the deficiencies are from Lindsley and Zimm (1992). pjc-16 maps between the markers px (58E3-10) and sp (60C1-6).

b Indicates an ems allele.

c Indicates an X-ray allele.
Figure 2.—Modification of the GMR-dE2FdDPp35 phenotype by enhancer mutations. Scanning electron micrographs of eyes from the following genotypes: GMR-dE2FdDPp35/1 (A), GMR-dE2FdDPp35/+, E(E2F)2Aksh-4 (B), GMR-dE2FdDPp35/+, E(E2F)2Bpjc-10 (C), GMR-dE2FdDPp35/++, E(E2F)3Aksh-6/1 (D), GMR-dE2FdDPp35/++, E(E2F)3Bksh-9/1 (E), GMR-dE2FdDPp35/++; E(E2F)3C E65/1 (F), and GMR-dE2FdDPp35/++; E(E2F)3Dpjc-5/1 (G). Magnification × 200.

p35 in the background. Alleles of E(E2F)2A were strong enhancers of the GMR-dE2FdDP phenotype, whereas alleles of the other five groups were weak enhancers when p35 was absent (Figure 3).

**Trithorax group mutations:** E(E2F)3A mutations mapped between the markers hairy (h) and thread (th) on the third chromosome. No recombinants between E(E2F)3A and th were isolated out of 49 events, suggesting that E(E2F)3A maps close to th. Complementation tests with deficiencies in that region were done and one deficiency, Df(3L)brm11 (71F3-5–72D1-5), failed to complement all four of the E(E2F)3A mutations. Candidate mutations within that interval were tested for their ability to complement E(E2F)3A mutations and to enhance the GMR-dE2FdDPp35 phenotype. A mutation in the gene brhma, brm9, failed to complement all E(E2F)3A alleles and enhanced the GMR-dE2FdDPp35 phenotype, suggesting that E(E2F)3A alleles are new alleles of brhma. Interestingly, the Df(3L)brm11 mutation had no effect on the GMR-dE2FdDPp35 phenotype. However, another deficiency in that region, Df(3L)th102 (71F3-5–72D12), did enhance the GMR-dE2FdDPp35 phenotype, suggesting that the lack of enhancement by Df(3L)brm11 might be due to other mutations in the stock.

brm is a member of a class of genes called the trithorax group (Kennison 1993, 1995). Mutations in the trithorax group of genes were originally isolated as dominant suppressors of Polycomb mutations (Kennison and Tamkun 1988). The Polycomb group of proteins repress the expression of homeotic genes by locally modifying chromatin structure and silencing gene expression. The trithorax group genes are thought to activate homeotic gene expression by directly opposing the silencing effect...
of the Polycomb group. Some of the trithorax group genes, like brm, are thought to reverse the effects of Polycomb group silencing by directly affecting chromatin structure. The brm gene has been cloned and encodes a putative ATPase with homology to the SWI2/SNF2 family of proteins in yeast and humans (Tamkun et al. 1992). SWI/SNF proteins function in large protein complexes that affect gene expression by modifying chromatin structure (Carlson and Laurent 1994; Peterson and Tamkun 1995). Likewise, the BRM protein exists in a large protein complex in Drosophila embryo extracts (Papoulas et al. 1998).

Because BRM functions in a protein complex, it is possible that we isolated mutations in other members of this complex in the GMR-de2FdDPp35 screen. Two of the enhancer groups, E(E2F)3B and E(E2F)3C, map very close to the trithorax group mutations, moira (mor) and osa, respectively (Kennison and Tamkun 1988; Goldman-Levi et al. 1996). Alleles of moira (mor1, mor2, and mor3) enhance the GMR-de2FdDPp35 phenotype and fail to complement both E(E2F)3B mutations. Likewise, an osa1 allele enhances the GMR-de2FdDPp35 phenotype and fails to complement all five E(E2F)3C alleles. Mutations in other trithorax and Polycomb genes tested, including Brista (Ba or Dll), extra sex combs (esc), pleiohomeotic (pho), Polycomb (Pc), trithorax (trx), Polyclobleike (Pcl), Sex combs on midleg (Scm), super sex combs (sxc), devener (dev), kohtalo (kto), and kismet (kis), had little or no effect on the GMR-de2FdDPp35 phenotype, suggesting that the effect is specific to only certain members of the trithorax class of genes (data not shown).

Several lines of evidence show that the functions of brm, mor, and osa are closely interconnected. Alleles of brm and mor were both isolated as suppressors of Pc (Kennison and Tamkun 1988) and have similar effects on the expression of Ubx and engrailed (Brizuela et al. 1994; Brizuela and Kennison 1997; Elfving et al. 1998). Allele-specific interactions have been reported between brm and mor (Papoulas et al. 1998). mor encodes a Drosophila homolog of the yeast SWI3 protein and the human BRG1/hBRM-associated factors BAF155 and BAF170 (Crosby et al. 1999). Immunochemical experiments show that MOR binds to BRM (Crosby et al. 1999) and is a stoichiometric component of purified BRM complexes (Papoulas et al. 1998). The recent cloning of osa revealed that it is identical to eyed (Vázquez et al. 1999). OSA contains an ARID DNA-binding domain, which is also present in yeast SWI1. osa interacts genetically with brm and is required for the expression of some, but not all, of the brm-regulated homeotic genes (Vázquez et al. 1999). OSA was not a stoichiometric component of purified BRM complexes (Papoulas et al. 1998) and has been proposed to target BRM complexes to a subset of promoters (Vázquez et al. 1999).

pointed (pnt): E(E2F)3D mutations mapped approximately five map units distal to the marker ebony on the third chromosome, which places E(E2F)3D around 94A-94E. One possible candidate in that region was pointed (pnt) because mutations in pnt have been shown to alter eye development (Brunner et al. 1994; O’Neill et al. 1994). Alleles of pnt (pnt0548433 and pnt0785478) fail to complement all three E(E2F)3D mutations, suggesting that E(E2F)3D mutations are new alleles of pointed. Surprisingly, pnt0548433 did not enhance the GMR-de2FdDPp35 phenotype and pnt0785478 only weakly enhanced the GMR-de2FdDPp35 phenotype. It is possible that our alleles are stronger loss-of-function alleles or that a specific change in the pointed protein is required to affect the E2F phenotype. The PTD gene product is a member of the ETS family of transcription factors (Klimb 1993) and functions as a positive nuclear effector in the sevenless and epidermal growth factor receptor (EGF-R) signal transduction cascades (Brunner et al. 1994; O’Neill et al. 1994; Gabay et al. 1996). Like other components of the EGF and sevenless pathways, PNT is required for proper photoreceptor cell differentiation. Clones of strong pnt alleles in the eye frequently lack from one to three photoreceptor cells in the majority of ommatidia (Brunner et al. 1994; O’Neill et al. 1994).
If signaling via the sevenless or EGF-R pathway affects E2F activity, then other members of the pathway, in addition to ptd, might show a genetic interaction with GM R-dE2FdDPp35. It is possible that mutations in other pathway components were not isolated because the GM R-dE2FdDPp35 screen was not saturated. We tested mutations in Ras1, Raf, EGF-R, yan, the rolled map kinase, and Gap1 for their ability to enhance or suppress the GM R-dE2FdDPp35 phenotype. Ellipse, a gain-of-function mutation in EGF-R; Sevenmaker (Smm), a gain-of-function mutation in the rolled map kinase, and loss-of-function mutations in Ras1 were dominant enhancers of GM R-dE2FdDPp35 (data not shown). Ellipse mutations have a dominant eye phenotype, making it difficult to distinguish between an additive or synergistic interaction with the GM R-dE2FdDPp35 rough eye phenotype. None of the other mutations tested (see materials and methods) enhanced or suppressed GM R-dE2FdDPp35.

polycephalon (poc): The largest complementation group mapped to the left arm of the second chromosome distal to the marker aristale. All of the mutations were uncovered by Df(2L)PM F, indicating that E(E2F)2A was located between 21A and 21B7/8. Previously identified mutations within this interval were tested with E(E2F)2A alleles and both E(Raf)2A alleles and polycephalon (poc) alleles failed to complement all seven E(E2F)2A mutations. poc alleles also failed to complement E(E2F)2A alleles, suggesting that E(E2F)2A, poc, and E(Raf)2A are all mutations in the same gene. Throughout the rest of the article this gene is referred to as poc except when a particular allele is described. E(Raf)2A mutations were isolated as dominant enhancers of an activated Raf rough eye phenotype (Dickson et al. 1996). poc mutations were isolated as dominant enhancers of hypomorphic mutations in Deformed (Dfd), a homeotic gene required for the development of head structures (Gellon et al. 1997). poc mutant embryos die during embryogenesis with head deformities and weak segmentation defects (Dickson et al. 1996; Gellon et al. 1997). poc also functions later in development. Clones of E(Raf)2A alleles in the eye are small, suggesting that poc is required for cell viability or cell proliferation (Dickson et al. 1996). The clones also have variable numbers of photoreceptors, indicating a potential role for poc in photoreceptor development (Dickson et al. 1996).

poc mutations have one characteristic that sets them aside from the other five groups of enhancer mutations isolated in the screen. Alleles of poc clearly enhance the GM R-dE2FdDP phenotype without GM R-p35 in the background. With the exception of the E65 mutation in osa, all other enhancer mutations had little effect on the GM R-dE2FdDP phenotype unless p35 was used to eliminate E2F-induced apoptosis. GM R-dE2FdDP eyes heterozygous for poc mutations are rougher and have more bristles than GM R-dE2FdDP eyes (Figure 3).

BrdU incorporation was used to test the model that poc mutations might increase the number of ectopic S phases caused by elevated E2F expression (Figure 4). No large changes in BrdU incorporation were seen in postmitotic cells; however, the second wave of S phases was abnormal in GM R-dE2FdDP, +/+ poc- eye disks. In wild-type (Figure 4A) and GM R-dE2FdDP/+ disks (Figure 4B) there is a gradient in the intensity of staining in the second mitotic wave. Anterior cells in the stripe (right) stain darker than cells in the posterior half of the stripe (left). In GM R-dE2FdDP, +/+ poc- eye disks the gradient is altered and both posterior and anterior cells stain intensely (Figure 4C). The E2F target gene RNR2 is expressed in a stripe that roughly coincides with the second mitotic wave (A. Brook and N. Dyson, unpublished results). Despite the change in DNA synthesis, we saw no alteration in the expression of RNR2 in GM R-dE2FdDP eye disks heterozygous for poc mutations.

The above results suggest that poc mutations affect cell proliferation but independently of RNR2 expression. To further characterize the function of poc in cell-cycle control we determined whether poc mutations could enhance or suppress other cell cycle phenotypes. Expression of human p21Cip1 under the control of the GMR promoter suppresses S-phase entry posterior to the furrow and completely abolishes the second mitotic wave (de Nooij and Hariharan 1995). Differentiation occurs normally but with fewer than the normal number of cells, resulting in a depletion in the pool of undifferentiated cells before the later cell types have formed. This cell shortage causes the compound eyes of GM R-
p21 flies to appear rough. poc alleles suppress the GM R-p21 phenotype, restoring the eye morphology (Figure 5, A and B). A fly homologue of p21CIP1, dacapo (de Nooij et al. 1996; Lane et al. 1996), causes a similar phenotype when expressed from the GMR promoter. Because GMR-dacapo flies have a mild phenotype, we tested the effect of poc mutations on a GMR-dacapo fly heterozygous for a loss-of-function cyclin E mutation. Cyclin E mutations are dominant enhancers of the GMR-dacapo phenotype and cause a more severe rough eye phenotype where suppression can be more easily scored (de Nooij et al. 1996). poc mutations suppressed the phenotype of cycE185, GMR-dacapo/ + flies and restored the eye morphology (Figure 5, C and D).

To investigate how poc alleles suppress the GM R-p21, the pattern of BrdU-incorporating cells was examined in GMR-p21 eye disks with and without one copy of a poc allele (Figure 6). In the majority of eye disks examined the second mitotic wave was partially restored, indicating that suppression is due to an increase in cell proliferation. Thus, the level of POC appears to be important for p21CIP1 to prevent S-phase entry.

**DISCUSSION**

The studies of mammalian E2F and RB proteins face two major problems. First, the system is very complicated. The term “E2F” refers to the collective activity of many different E2F proteins and E2F complexes. Biochemical studies have shown that these have similar properties and, most likely, overlapping functions. Second, it is clear that E2F-dependent transcription can be
regulated at many different levels and pRB phosphorylation represents only one step of an intricate series of events leading to the activation of E2F. The expression of E2F target genes is accompanied by increased synthesis of specific E2Fs (most notably E2F-1, E2F-2, and E2F-3), by changes in subcellular localization of E2F-4 complexes, by protein phosphorylation, and by the appearance and disappearance of specific combinations of E2F/pRB family complexes (reviewed in Dyson 1998). Many of these changes likely contribute to the activation of transcription. Their effects can be demonstrated in carefully designed assays, but it is difficult to tell which types of regulation, and which of the many activities of E2F or RB proteins, are important for biological function in vivo.

Here we report the first attempt to identify, in a relatively unbiased way, genes that functionally interact with E2F. We have identified two loci on the second chromosome and four loci on the third chromosome that enhanced the GMR-dE2FdDPp35 phenotype. No suppressor mutations were recovered, suggesting that the GMR-dE2FdDPp35 phenotype is not sensitive to dominant-suppressing mutations or that the screen was not saturated. Likely suppressors of the GMR-dE2FdDPp35 phenotype are loss-of-function mutations in positive regulators of the cell cycle and gain-of-function mutations in negative regulators of the cell cycle. Mutations in E2F target genes could also be isolated as suppressors if the product of the target gene were limiting. We know from our preliminary analysis of the GMR-dE2FdDPp35 phenotype that loss-of-function mutations in the cyclins or cdk5 do not suppress the phenotype, suggesting that this phenotype may not be sensitive to mutations in positive upstream regulators. Overexpression of RBF, a negative regulator of E2F activity, does suppress the GMR-dE2FdDPp35 phenotype; however, gain-of-function mutations in negative regulators would be rare and probably were not recovered in the numbers screened.

Three of the loci on the third chromosome are new mutations in brm, mor, and osa, three trithorax group genes. Although the trithorax group is quite large, no mutations were identified in other members of this group. Alleles of other trithorax and Polycomb group mutations failed to affect the GMR-dE2FdDPp35 phenotype, indicating that the enhancement of the GMR-dE2FdDPp35 phenotype is not due to global deregulation of homeotic gene expression. The BRM protein is part of a large protein complex that is believed to modify chromatin structure (Dingwall et al. 1995; Papoulas et al. 1998). Allele-specific genetic interactions have been reported between brm, mor, and osa and recent reports show that BRM complexes contain MOR and may also physically interact with OSA (Papoulas et al. 1998; Crosby et al. 1999; Vázquez et al. 1999). Taken together these results suggest that the activity of a chromatin-remodeling complex containing BRM, MOR, and OSA has an important impact on the GMR-dE2FdDPp35 phenotype.

Initial studies of BRM showed that it can assist transcriptional activation. More recent work shows that SWI/SNF complexes remodel nucleosome structure, facilitating an exchange between a normal structure and an alternative conformation in which the DNA is more accessible to transcription factors (Schnitzler et al. 1998). In this way BRM complexes can facilitate DNA binding by a variety of factors that can either activate or repress transcription. The genome-wide expression analysis of Saccharomyces cerevisiae SWI2 mutants suggests that only a small percentage of genes depend on the ability of the SWI/SNF complexes to remodel chromatin (Holstege et al. 1998). Surprisingly, more genes appear to be negatively regulated by SWI2 than are positively regulated (Holstege et al. 1998).

The molecular basis of the interaction between E2F and a BRM/MOR/OSA chromatin-remodeling complex is not yet clear and a range of possibilities exist. The genetic interaction may result from a direct physical interaction between RBF/E2F complexes and chromatin-remodeling machinery. In support of this idea the human homologues of BRM, hBRM, and BRG1 have been found to physically associate with pRB (Dunaief et al. 1994; Singh et al. 1995). This raises the possibility that BRM/MOR/OSA may help E2F/RBF repressor complexes bind to their target sites. This interpretation is supported by experiments from Trouche and co-workers who used transient transfection of mammalian cells to demonstrate that BRG1 can cooperate with pRB to repress E2F-dependent transcription (Trouche et al. 1997). Consistent with this model, the introduction of two copies of GMR-RBF into a GMR-dE2FdDPp35/+; brm+/+ background suppressed the enhancement by brm. Thus the effect caused by low levels of brm could be overcome by increasing the dosage of RBF (data not shown). We have searched for additional evidence that would be predicted by this model but, to date, these experiments have been inconclusive. BRM lacks the LXCXE motifs found in hBRM and BRG1, which have been suggested to mediate the interaction with pRB (Dunaief et al. 1994; Singh et al. 1995). To date we and others (O. Papoulas and J. W. Tamkun, personal communication) have failed to detect a physical interaction between BRM and RBF or between BRM and dE2F. The interaction between endogenous pRB and hBRM or BRG1 proteins is hard to detect even in mammalian cells, and our failure to find BRM/RBF complexes may simply reflect difficulty in extracting chromatin-associated proteins under conditions that maintain the interaction.

An alternative possibility is that the BRM/MOR/OSA chromatin-remodeling complex is an important regulator of the expression of some key E2F-target genes, but this complex does not interact directly with either RBF or E2F. In this case the functional interaction occurs because these proteins converge on overlapping sets of promoters. This model is difficult to test because it is not yet clear which, and how many, E2F target genes
are functionally significant. RNR2, one example of an E2F-dependent gene, is expressed normally in embryos mutant for brm, osa, or mor and we have been unable to detect any change in the expression of RNR2 in GM-R-deE2FdDP35 eye disks heterozygous for brm, osa, or mor alleles. While RNR2 expression is often used to provide an in vivo readout of E2F activity, experiments suggest that it is not a critical E2F target (Royzman et al. 1997; Du and Dyson 1999). The effects of brm, mor, and osa may only be evident at a subset of E2F-regulated promoters and an extensive screen of E2F targets will be necessary to find the appropriate gene.

Finally, it is possible that E2F and brm act in distinct pathways that influence cell-cycle progression. In this model the activity of a BRM/MOR/OSA-containing complex may have a function that influences the ability of E2F or RBF to control S-phase entry. Several observations have linked BRM-related proteins to cell-cycle control. brm null clones in the adult cuticle often show duplications of bristle structures, suggesting a possible role for brm in proliferation (Elfring et al. 1998), and mice lacking the BRM homolog SNF2 show evidence of increased cell proliferation (Reyes et al. 1998). Although brm, mor, and osa had no effect on the GMR-p21 phenotype (data not shown), both brm and mor mutations were recently isolated as suppressors of a hypomorphic cyclin E eye phenotype, demonstrating that brm and mor can affect other cell-cycle phenotypes in the eye (H. Richardson, personal communication). Other studies have shown that the activity of hSWI/SNF complexes is itself cell-cycle regulated (Sif et al. 1998). Transformation by activated Ras decreased the expression of the murine ortholog of hBRM in mouse fibroblasts (Muchardt et al. 1998), whereas growth arrest led to an accumulation of protein (Muchardt et al. 1998). Recently, BRG1 and BAF155, a human ortholog of Moira, were shown to associate with cyclin E and were suggested to be targets for cyclin E-dependent kinases during S-phase entry (Shanahan et al. 1999).

During this study we observed that GMR-dE2FdDP p35/+; brm-/+ eyes developed necrotic patches that increased in severity with the age of the adult fly. This raised the possibility that brm mutations might enhance the phenotype by promoting E2F-induced apoptosis. However, further experiments failed to support this hypothesis. brm mutations failed to enhance the GMR-dE2FdDP phenotype that has elevated levels of apoptosis or to modify a GMR-rpr phenotype. In addition, brm mutations had no effect on the phenotype of animals in which GMR-rpr and GMR-hid-induced apoptosis is blocked by GMR-p35 (data not shown). No increase in the number of apoptotic cells was detected when GMR-dE2FdDP35/+; brm-/+ third instar eye disks were stained with acridine orange (data not shown).

The identification of pnt, Ras1, and rolled mutations as enhancers of an E2F overexpression phenotype suggests that there might be crosstalk between the signaling pathways downstream of receptor tyrosine kinases and the E2F pathway. The basis for this interaction is not clear and may be complex because both loss-of-function mutations (in Ras1 and pnt) and a gain-of-function mutation (in the rolled MAP kinase) enhance the E2F-dependent phenotype.

Of all the complementation groups isolated in this screen, polycephalon showed the most extensive effects on cell-cycle phenotypes. Poc mutations enhanced the GMR-dE2FdDP phenotype in the absence of p35 and suppressed the GMR-rpr21 phenotype. Poc was also the only group of enhancer mutations that could be clearly shown to alter the pattern of S phases in the eye imaginal disk. Partial restoration of the second wave of S phases in GMR-p21, +/+ poc- eye disks indicates that the level of poc is important for p21cip1-mediated cell cycle arrest. Mutations in poc also altered the pattern of S phases in the second mitotic wave in eye disks of GMR-dE2Fd DP larvae. Unlike wild-type and GMR-dE2FdDP/+ eye disks, in GM R-dE2FdDP, +/+ poc- eye disks the intensity of BrdU staining did not drop off at the posterior of the second wave but instead remained elevated throughout. Other mutations have been reported to alter the second mitotic wave. In roughex (rux) mutants cells enter S phase prematurely and as a result the domain of S phases is expanded anteriorly toward the furrow (Thomas et al. 1994). To our knowledge this is the first report of a mutation that affects the posterior domain of the second wave. A detailed investigation of the effect of poc on the cell cycle awaits the cloning of this gene.

This study demonstrates the use of a genetic screen in Drosophila to isolate genes that functionally interact with the E2F transcription factor. Four of six genes identified are known to regulate gene expression, and three encode components of a chromatin-remodeling complex. These results add to the emerging view that chromatin conformation is a key feature of E2F regulation and suggest that SWI/SNF complexes play an important role either in E2F regulation or in the control of S-phase entry. We note that this screen is not saturated and that many additional E2F interactors remain to be identified. Future studies will also be needed to identify the precise molecular basis for these genetic interactions and to determine whether the human counterparts of osa, moira, pnt, and poc also regulate E2F activity in mammalian cells.

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LITERATURE CITED


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