Two mRNA species were observed for the Drosophila E2F (dE2F) gene, differing with regard to the first exons (exon 1-a and exon 1-b), which were expressed differently during development. A single transcription initiation site for mRNA containing exon 1-b was mapped by primer extension analysis and numbered +1. We found three tandemly aligned sequences, similar to the DNA replication-related element (DRE; 5′-TATCGATA), which is commonly required for transcription of genes related to DNA replication and cell proliferation, in the region upstream of this site. Band mobility shift analyses using oligonucleotides containing the DRE-related sequences with or without various base substitutions revealed that two out of three DRE-related sequences are especially important for binding to the DRE-binding factor (DREF). On footprinting analysis with Kc cell nuclear extracts and a glutathione S-transferase fusion protein with the N-terminal fragment (1–125 amino acid residues) of DREF, all three DRE-related sequences were found to be protected. Transient luciferase expression assays in Kc cells demonstrated that the region containing the three DRE-related sequences is required for high promoter activity. We have established transgenic lines of Drosophila in which ectopic expression of DREF was targeted to the eye imaginal disc cells. Overexpression of DREF in eye imaginal disc cells enhanced the promoter activity of dE2F. The obtained results indicate that the DREF/DRE system activates transcription of the dE2F gene.

E2F is a transcription factor playing important roles in the regulation of cellular proliferation (1). In mammals, at least six distinct E2F family members (E2F1, E2F2, E2F3, E2F4, E2F5, and E2F6) and two heterodimer partners (DP1 and DP2) have been isolated (2–4). Many DNA replication-related mammalian genes such as those for DNA polymerase and E2F6) and two heterodimer partners (DP1 and DP2) have

1 The abbreviations used are: PCNA, proliferating cell nuclear antigen; DRE, DNA replication-related element; DREF, DRE-binding factor; CFDD, common regulatory factor for DNA replication and DREF sites in their promoters. Transcription of these genes is induced by E2F family members during progression from G0 to S phase (2).

In general, the levels of DP1, DP2, and E2F4 proteins are constant throughout the cell cycle (5, 6), whereas dramatic increase in expression of E2F1 and E2F2 genes during progression from G0 to S phase has been reported (7, 8). The mechanisms of transcriptional regulation have been studied (8–10), and E2F1 and E2F2 genes contain E2F-binding sites in their promoters, their expression being repressed by E2F-mediated negative control through these sites during G0 phase (8–11). The repression is likely mediated by an E2F-Rb family complex. In late G1 phase, E2F-mediated repression is relieved by G1 cyclin-dependent kinase-mediated phosphorylation of a Rb family protein (12). Then, transcription factors like CCAAT-binding protein and YY-1 appear to activate the E2F1 gene promoter (11, 13), while transcription factor Myc activates the E2F2 gene promoter (8).

In Drosophila, a single set of E2F and DP has been identified and characterized (14, 15), but two different E2F cDNAs have been reported (14, 15). Although these two cDNAs encode an identical protein, the nucleotide sequences in their 5′-untranslated regions differ.

Comparison of the newly determined genomic sequences of the Drosophila E2F gene (dE2F) and its 5′-flanking region2 with the cDNAs nucleotide sequences allowed us to map two genomic regions, corresponding to the diverged sequences of two E2F cDNAs. These two genomic regions likely represent parts of two different first exons, named 1-a and 1-b, that are, respectively, located 10.1 and 5.4 kb upstream of the common second exon containing the translation initiation codon. Northern hybridization analyses using specific probes confirmed the existence of two transcripts, named transcripts-a and -b. Although transcript-a was detected exclusively in embryos, transcript-b could be identified in other stages of development. Fluctuation of transcript-b level was roughly similar to those of other DNA replication-related genes (16, 17). Thus, we were interested in knowing whether synthesis of transcript-b is regulated by the same factors involved in expression of DNA replication-related genes.

Like their mammalian counterparts, Drosophila E2F and DP form a heterodimer to generate a sequence-specific DNA binding factor that appears to activate transcription of DNA replication-related genes through E2F-binding sites (14, 15, 18–22).
In addition to E2F-binding sites, a common 8-base pair (bp) palindromic sequence named the DNA replication-related element (DRE; 5′-TATCGATA) is frequently found in the promoters of growth-related genes (23–27). A transcription factor DREF (DRE-binding factor) that specifically binds to the DRE sequence has been isolated as an 80-kDa polypeptide homodimer (23), and its cDNA has been cloned (28). Our previous studies performed in vitro and in vivo suggested that E2F-binding sites and DRE function synergistically to activate promoters of the PCNA and DNA polymerase α 180-kDa subunit genes (23, 29, 30).

Interestingly, we found three tandemly aligned DRE-related sequences, each of which matched 6 bp out of the 8-bp DRE consensus sequence, located at positions −540 to −533 (site I), −532 to −525 (site II), and −521 to −514 (site III) with respect to the initiation site of transcript-b. The region from −2184 to −203 showed promoter activity in Drosophila Kc cells with the three DRE-related sequences playing an important role. In addition, DREF specifically bound to these DRE-related sequences, and overexpression of DREF in Drosophila eye imaginal disc cells activated the expression of the lacZ reporter gene, which was under the control of the dE2F gene promoter. The obtained results indicate that transcription of the dE2F gene is regulated by the DRE/DREF system.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**Kc cells derived from Drosophila melanogaster embryos were grown at 25 °C in M3/FB medium (31) supplemented with 2% fetal calf serum in the presence of 5% CO₂.

**Antibodies—**Monoclonal antibodies to DREF, mAb 1 and mAb 4, were raised as described previously (28).

**Isolation of Drosophila Genomic Clones—**A genomic library was constructed by Sau3A1 partial digestion of D. melanogaster Oregon-R DNA and ligation into the BamHI site of λEMBL3. The details of the procedures for screening and nucleotide sequencing of the genomic clones for dE2F will be described elsewhere (accession number AB011813).

**Oligonucleotides—**To determine the transcription initiation site, the following oligonucleotide was used in primer extension experiments: primer 4.5pet, 5′-AATCTTTTACCTGCAGAGGCAGTGTTTTGCGGCT-3′.

To obtain a fragment containing the promoter of the dE2F gene, the following polymerase chain reaction (PCR) primers were chemically synthesized: −9610HS2, 5′-CTCCAGCCTCCGCGGGGAAGATGGTGAAGCGGTT; and −8230X, 5′-AACTCGAGGAAGATGGTGAAGCGGTT; and +8771, 5′-TTTCTCCGAGAACACTTATGATTAGGCGT; and +8771, 5′-TTTCTCCGAGAACACTTATGATTAGGCGT.

To obtain a fragment containing the first exons of the dE2F gene, the following PCR primers were synthesized: 5.2ss3761, 5′-AATCTTTTACCTGCAGAGGCAGTGTTTTGCGGCT-3′; and 5.3AI partial digestion of Oregon-R DNA and ligation into the BamHI site of pBluescript II SK(−) and then digested with the blunt-ended BglII site of the plasmid PGVB. To construct the plasmid p-440E2Fbluc, plasmid DPA124SE4.5 was digested with DraI and EcoRV, and the DNA fragment containing the dE2F gene fragment was isolated and inserted into the blunt-ended BglII site of the plasmid PGVB.

To construct the plasmid p-6132E2Fbluc, PCR was performed using plasmid DPA124SE4.5 as a template, and primers +8230X and +9610HS2 in combination. The PCR products were digested with XhoI and HindIII and inserted between the XhoI and HindIII sites of plasmid pGMR. To construct the plasmid p-342E2Fbluc, plasmid DPA124SE4.5 was digested with DraI and EcoRV, and the DNA fragment containing the dE2F gene fragment was isolated and inserted into the blunt-ended BglII site of the plasmid PGVB.

To construct the luciferase expression plasmid having three DRE-related sequences placed upstream of the heterologous TATA promoter (Fig. 9), the oligonucleotides DRE-EB or DRE-EB (mut I II III) were ligated in a head-to-tail manner as described previously (23). The ligated oligonucleotides were isolated and digested with DRE and BamHI, and inserted into the BamHI site of the plasmid p-TATA-SK. The fragments containing TATA and DRE promoters were isolated by digestion with Smal and SacI, and inserted between Smal and SacI sites of PGVB. For p-TATA-SK, TATA-CAT (23) was digested with XhoI, after which the DNA fragment containing metallothionein basal promoter was isolated, and inserted into the XhoI site of plasmid pBluescript II SK(−). To construct the control plasmid TATA-PGVB carrying the metallothionein basal promoter alone, TATA-SK was digested with KpnI and SacI, and the DNA fragment containing the TATA promoter was isolated and inserted into the blunt-ended BglII site of the plasmid PGVB.

To construct the plasmid p3x8SF-pBluescript II SK(−) for the footprinting analysis, plasmid DPA124SE4.5 was digested with PvuII, and the DNA fragment containing the region from −843 to −440 was isolated. Then, this fragment was inserted into the EcoRV site of the pBluescript II SK(−).

To construct the plasmid p-2184E2Fbluc for the luciferase-transient expression assay, pActCAT was digested with SacI and HindIII, and the DNA fragment that contained the actin 5C gene promoter was isolated and blunt-ended using T4 DNA polymerase. Then, this DNA fragment was inserted into Ehel site of the plasmid pRL-null vector (Promega).

To construct the plasmid p-3x8E/S-pBluescript II SK(−) for the footprinting analysis, plasmid DPA124SE4.5 was digested with PvuII, and the DNA fragment containing the dE2F gene fragment was isolated and inserted into the blunt-ended BglII site of the plasmid PGVB. To construct the plasmid p-11592E2Fbluc, PCR was performed using plasmid DPA124SE4.5 as a template, and primers +8230X and +9610HS2 in combination. The PCR products were digested with XhoI and HindIII and inserted between the XhoI and HindIII sites of plasmid pGMR. To construct the plasmid p-9132E2Fbluc, plasmid DPA124SE4.5 was digested with DraI and EcoRV, and the DNA fragment containing the dE2F gene fragment was isolated and inserted into the blunt-ended BglII site of the plasmid PGVB. To construct the plasmid p-6132E2Fbluc, PCR was performed using plasmid DPA124SE4.5 as a template, and primers +8771 and +9610HS2 in combination. The PCR products were digested with XhoI and HindIII and inserted between the XhoI and HindIII sites of plasmid pGMR.

To construct the plasmid p-440E2Fbluc, plasmid DPA124SE5.4 was digested with PvuII, and the DNA fragment containing the dE2F gene fragment was isolated and inserted into the blunt-ended BglII site of the plasmid PGVB. To construct the plasmid p-342E2Fbluc, plasmid DPA124SE4.5 was digested with Nael and EcoRV, and the DNA fragment containing the dE2F gene fragment was isolated and inserted into the blunt-ended BglII site of the plasmid PGVB.

To construct the luciferase expression plasmid having three DRE-related sequences placed upstream of the heterologous TATA promoter (Fig. 9), the oligonucleotides DRE-EB or DRE-EB (mut I II III) were ligated in a head-to-tail manner as described previously (23). The ligated oligonucleotides were isolated and digested with DRE and BamHI, and inserted into the BamHI site of the plasmid p-TATA-SK. The fragments containing DRE and TATA promoter were isolated by digestion with Smal and SacI, and inserted between Smal and SacI sites of PGVB. For p-TATA-SK, TATA-CAT (23) was digested with XhoI, after which the DNA fragment containing metallothionein basal promoter was isolated, and inserted into the XhoI site of plasmid pBluescript II SK(−). To construct the control plasmid TATA-PGVB carrying the metallothionein gene basal promoter alone, TATA-SK was digested with KpnI and SacI, and the DNA fragment containing the metallothionein gene basal promoter was isolated and inserted into the KpnI and SacI sites of PGVB.

To construct the plasmid pGM-RAL-GAL, PCR was performed using plasmid pGEMT as a template, and primers GCGCCGCTCAT and CTATATAG in combination. The PCR products were digested with EcoRI and BglII, and inserted between the EcoRI and BglII sites of plasmid pGM-R. To construct the pUAS-DREF, PCR was performed using plasmid DC-DREF2.2 (23) as a template, and primers DREF-ATG and T7 primer (Toyobo) in combination. The PCR products were digested with BamHI and Xhol, and then inserted between BamHI and Xhol sites of pUAST (33).
Transcriptional Regulation of the Drosophila E2F Gene

Fig. 1. Genome and transcript organization of the two cDNAs for Drosophila E2F. A, genomic map of the dE2F locus. The positions of exon 1-a and 1-b specific probes for the Northern hybridization analysis are shown. In the dE2F mutant, dE2F729, the P-element carrying the lacZ is inserted 48 nucleotides upstream of the initiator methionine. S, Sall; E, EcoRI; B, BamHI; H, HindIII. B, the structures of the two of dE2F cDNAs reported previously (14, 15). The open boxes represent untranslated regions, and the coding regions are shown by the shaded boxes. The introns are indicated by thin lines.

All plasmids were propagated in Escherichia coli XL-1 Blue, isolated by standard procedures (34) and further purified through two cycles of ethidium bromide/CsCl density-gradient centrifugation.

Northern Blot Hybridization Analysis—Total cellular RNA was isolated from bodies of Drosophila at various developmental stages by the acid guanidium thiocyanate-phenol-chloroform extraction method (35). Twenty micrograms of total RNA were separated on a 1% agarose gel containing formaldehyde and blotted onto a sheet of GeneScreen Plus membrane (DuPont). Probes were radiolabeled using the random primer method (36). Hybridization and washing conditions were the same as described elsewhere (16). Blots were exposed to Kodak X-Omat XAR films or quantified with a BAS2000 (Fuji Film) imaging analyzer.

Fig. 2. Northern hybridization analysis using total RNA from Drosophila bodies at various developmental stages. Twenty micrograms of total RNA were applied to each lane. The radiolabeled probes were used successively on the same membrane. A, transcripts containing the region corresponding to exon 1-a are found for 0–2-h and 4–12-h embryos, while those containing the region corresponding to exon 1-b are apparent in the embryos, larvae, and pupae. Rp-49 mRNA served as loading control. B, amounts of transcript-a (closed circle) and transcript-b (open box) as determined using an imaging analyzer and expressed in relative values to the amount of the transcript in 4–8-h embryos. In the case of transcript-b, averaged values from two independent experiments are shown.

DNase I Footprinting Analysis—DNase I footprinting analysis was performed essentially as described previously (23). The DNA fragment (−440 to −843) was obtained by digestion of p-3xSBE-pBluescript II SK(−) with BamHI (lower) and HindIII (upper), labeled at 5′-end of the upper or lower strand. After electrophoresis, gels were dried and autoradiographed.

Fly Strains—Fly stocks were maintained at 25 °C on standard food. The Canton S fly was used as the wild type strain. The dE2F729 allele, described previously (19), was kindly supplied by Drs. A. Brook and N. Dyson.

Establishment of Transgenic Flies—P-element-mediated germ line transformation was carried out as described previously (38). F1 transformants were selected on the basis of white eye color rescue (39). Four independent lines were obtained with pGMR-GAl4 constructs and line 16 carrying pGMR-GAl4 on X chromosome was used. Five independent lines were obtained for pUAS-DREF constructs. The line carrying pUAS-DREF in the second chromosome was used in this study. The details of the procedures for establishment of lines with the UAS-DREF transgene will be described elsewhere.3

Immunohistochemistry—The line carrying GMR-GAl4 in the X chromosome was crossed with the line carrying UAS-DREF in the second chromosome, and the resultant hybrids were crossed with dE2F729. The progenies were analyzed as detailed below. Third instar larvae were dissected in Drosophila Ringer solution, and imaginal discs were fixed

3 F. Hirose, T. Sawado, M. Yamaguchi, Y. Nishi, K. Sakaguchi, and A. Matsukage, manuscript in preparation.
in 4% paraformaldehyde/PBS for 20 min at room temperature. After washing with PBS/0.3% Triton X-100 (PBS-T), the samples were blocked with PBS-T containing 10% normal goat serum for 30 min at room temperature. Samples were incubated with rabbit anti-DREF IgG at a 1:2000 dilution or with mouse anti-β-galactosidase monoclonal antibody at a 1:1000 dilution at 4 °C for 16 h. After extensive washing with PBS-T, the imaginal discs were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Promega) at a 1:1000 dilution or with peroxidase-conjugated goat anti-mouse IgG (E-Y Laboratory) at a 1:500 dilution as second antibody. After extensive washing with PBS-T, color was developed in a solution containing 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl₂, 0.34 mg/ml nitro blue tetrazolium salt, and 0.175 mg/ml 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt for the alkaline phosphatase reaction and in a solution containing 0.5 mg/ml diaminobenzidine, 2.5 mM CoCl₂, and 0.003% H₂O₂ for the peroxidase reaction. The tissues were washed with PBS and mounted in 90% glycerol/PBS for microscopic observation.

**RESULTS**

Two Transcripts of Drosophila E2F Are Possibly Synthesized by Different Mechanisms during Development—Two different cDNAs of *Drosophila* E2F have been reported (14, 15). Although these two cDNAs encode an identical protein, the nucleotide sequences in their 5'-untranslated regions are divergent. Comparison of the newly determined genomic sequence of dE2F gene and its 5'-flanking region with those of cDNAs allowed us to map two genomic regions corresponding to the divergent sequences of the two E2F cDNAs. These two genomic regions likely represent parts of two different first exons, named 1-a and 1-b, that are, respectively, located 10.1 and 5.4 kb upstream of the common second exon containing the translation initiation codon (Fig. 1).

We examined the dE2F mRNA level using total RNA from *Drosophila* bodies at various developmental stages (Fig. 2). Northern hybridization analysis using the exon 1-b specific probe detected on an approximately 4.2-kb transcript (transcript-b) at a relatively low level in unfertilized eggs and 0–2-h embryos (Fig. 2). Transcript-b was detectable throughout developmental stages, although its size appeared slightly larger in 4–24-h embryos, larvae, pupae, and adult flies (Fig. 2 and FIG. 3.

**Fig. 3.** Determination of the transcription initiation site of transcript-b by the primer extension method. 32P-Labeled 34-mer primer (see Fig. 4) complementary to the 5'-part of the cDNA reported by Dynlacht et al. (15) was hybridized with total RNA isolated from unfertilized eggs (lane 5), 8–12-h embryos (lane 6), 3rd instar larvae (lane 7), and Kc cells (lane 8). The primer was extended using reverse transcriptase as described under “Experimental Procedures.” To align the extended products with the genomic sequence, a parallel dideoxy-sequencing reaction was carried out using the same 34-mer primer (lanes 1–4). The numbers on the right indicate the nucleotide positions from the transcription initiation site, defined as +1.

![Fig. 3](image-url)
In contrast, the exon 1-a specific probe detected a transcript of 4.7 kb (transcript -a) at highest levels in 4–8-h embryos, and only at low levels in 0–4-h and 8–12-h embryos. Transcript-a was not detectable in unfertilized eggs, late stages of embryogenesis, larvae, and pupae. Since both the regions corresponding to the first exons are short, the signals detected by both probes were rather weak. The coding region specific probe detected both transcripts-a and -b (data not shown). A ribosomal protein 49 (Rp-49) cDNA was used as a reference probe to monitor the mRNA integrity. The results suggest that transcripts-a and -b are likely to be under the control of different regulatory mechanisms during development.

Determination of the Initiation Site of Transcript-b—Although the initiation site of transcript-a has been reported (40), that of transcript-b has hitherto not been determined. We therefore performed a primer extension analysis using a primer complementary to the 5’ region of exon 1-b (Fig. 3). Using total RNA from unfertilized eggs, 8–12-h embryos, third larvae, and Kc cells, a single transcription initiation site was mapped to 78 bp upstream from the 5’ end of the reported cDNA (15), and defined as nucleotide position +1. It should be pointed out that the same transcription initiation site was used for both the maternally transcribed mRNA (Fig. 3, lane 5) and the zygotically transcribed mRNA (Fig. 3, lanes 6–8).

As shown in Fig. 4, we found the three tandemly aligned
DRE-related sequences, with a 6 out of 8 bp DRE consensus match, located at positions -540 to -533 (site I), -532 to -525 (site II), and -521 to -514 (site III) with respect to the transcription initiation site. A potential E2F-binding site (TTTGC-CGG) was found at positions -41 to -34. Although a DRE-related sequence was found at -213 to -206, band mobility shift assays revealed that this DRE-related sequence was not recognized by DREF (data not shown).

**DREF Recognizes the Three DRE-related Sequences Located Upstream of the Initiation Site for Transcript-b**—To determine whether DREF can recognize the three DRE-related sequences located upstream of the initiation site of transcript-b, we performed band mobility shift assays using Kc cell nuclear extracts (Fig. 5, A–C). DRE-Eb is the oligonucleotide containing three DRE-related sequences located upstream of the initiation site of transcript-b of the dE2F gene. DRE-P is the oligonucleotide containing DRE from the promoter region of the Drosophila PCNA gene (23), used as a control. DRE-PΔ3 is a 3-base-deletion derivative of the DRE-P (29).

With the radiolabeled DRE-P as a probe, a shifted band was observed (Fig. 5A, lanes 1, 5, and 9), which was diminished by adding excess unlabeled DRE-Eb (Fig. 5B, lanes 2–4) or DRE-P (Fig. 5B, lanes 6–8), indicating that a common binding factor could recognize both sequences specifically. In line with a previous study (29), DRE-PΔ3 did not compete for the binding (Fig. 5A, lanes 10–12). When the radiolabeled DRE-Eb was used as a probe, three to four protein-DNA complexes were formed with the Kc cell nuclear extract (Fig. 5B, lanes 1, 5, and 9). One of the shifted bands (Fig. 5B, arrow) was diminished by adding excess unlabeled DRE-Eb (Fig. 5B, lanes 2–4) and DRE-P (Fig. 5B, lanes 6–9), while the others demonstrated competition with DRE-Eb but not DRE-P. Again, DRE-PΔ3 did not compete for the binding (Fig. 5B, lanes 11 and 12).

To examine whether the shift bands observed with the DRE-Eb probe represent a DRE/DREF complex, we examined effect of the anti-DREF monoclonal antibodies (mAbs 1 and 4) on the binding reaction with Kc cell nuclear extracts. mAb 1 inhibited the binding of DREF to DRE-P, and mAb 4 supershifted the band with DRE-P (20, 28) (Fig. 5C, lanes 10–12). As shown in Fig. 5C, one of the shift bands with DRE-Eb probe was diminished by the addition of mAb 1 (lanes 2 and 3) and was supershifted by mAb 4 (lane 6). These results indicate that one of the DNA-protein complexes formed between DRE-Eb and Kc cell nuclear extracts contains DREF.

Using a recombinant fusion protein of GST and the N-terminal fragment (1–125 amino acid residues) of DREF (GST-DREF1–125), the band mobility shift assay was performed (Fig. 6, A and B). As noted in a previous study (28), the DNA binding domain resides in this N-terminal region and a DNA-protein complex was formed between DRE-P and GST-DREF1–125 (Fig. 6A). The addition of DRE-Eb or DRE-P to the binding reaction as competitors diminished the shifted band (Fig. 6A, lanes 1–8), while DRE-PΔ3 did not compete for the binding (Fig. 6A, lanes 9–12). When radiolabeled DRE-Eb was used as a probe, a DNA-protein complex was also formed with GST-DREF1–125 (Fig. 6B). This shifted band was also diminished by the addition of DRE-Eb or DRE-P (Fig. 6B, lanes 1–8), but not DRE-PΔ3 (Fig. 6B, lanes 9–12). Taking the results to-
Together, we conclude that DREF binds to the DRE-related sequences at −540 to −514 located upstream of the initiation site of transcript-b.

Role of the Three DRE-related Sequences in DREF Binding—To determine the nucleotide sequences in the DRE-Eb required for binding to DREF, a set of oligonucleotides having mutations in and around the three DRE-related sequences were chemically synthesized (Fig. 7A), and added to the binding reaction as competitors (Fig. 7B and C). With the 32P-labeled DRE-Eb, mutant oligonucleotide DRE-Eb (mut I) or DRE-Eb (mut IV) competed as effectively as wild type DRE-Eb (Fig. 6B, lanes 5–8 and 33–36). Mutant oligonucleotide DRE-Eb (mut II III) or DRE-Eb (mut I II III) did not compete for the binding (Fig. 6B, lanes 21–24 and 29–32). These results indicate that sites II and III in DRE-Eb are important for the binding to GST-DREF1–125.

Footprinting analyses with the Kc cell nuclear extracts and GST-DREF1–125 fusion protein demonstrated protection of the region from −540 to −514, which covers not only sites II and III but also site I (Fig. 8A and B), and the Kc cell nuclear extract also protected a similar region (Fig. 8A and C). The data provide evidence that the DREF-binding to site I is dependent on sites II and III.

In addition, it should be noted that another protection was observed with the Kc cell nuclear extract in the regions from −635 to −615 and −709 to −674, both containing a homodomain protein-binding consensus, TAAT (41) (Figs. 4 and 8A).

The Three DRE-related Sequences Are Required for High Promoter Activity of the dE2F Gene—Genomic fragments from −2184 to −203 of the dE2F gene containing the initiation site of transcript-b and its 5′-deletion derivatives were ligated with the luciferase reporter gene, and then the constructs were transfected into Kc cells to determine promoter activities (Fig. 9). Deletion from positions −2184 to −1159 resulted in 40% reduction of luciferase expression. A CFDD (common regulatory factor for DNA replication and DREF genes) recognition site, which is important for the promoter activity of the PCNA gene (42), was found at −1303 to −1299, although we have not yet confirmed whether this is responsible for the promoter activity. Deletion from −1159 to −913 decreased luciferase expression to 16%. Further deletions up to −613 did not show any additional significant change in luciferase expression. A deletion from −613 to −440 decreased luciferase expression to 1%, indicating the existence of strong positive regulatory elements within this region. As noted above, it contains the three DRE-related sequences (−540 to −514), sug-
Fig. 9. 5′-Deletion analysis of the promoter regions for transcript-b. Luciferase expression plasmid DNAs carrying upstream regions of various lengths were transfected into Kc cells, and firefly luciferase activity was measured. Schematic structures of the derivatives of the reporter plasmid are presented. The three DRE-related sequences are indicated by ovals. A potential CFDD-binding site (45) and the E2F-binding sites are indicated by shaded circles and shaded boxes, respectively. Open boxes indicate the homeodomain protein-binding consensus TAAT, protected in the Kc cell nuclear extract from DNase I digestion (Fig. 8A). Luciferase activities are expressed as percentages of the p-2184E2Fbluc value on the right.

Fig. 10. Effects of the three DRE-related sequences on the heterologous promoter. Luciferase expression plasmids having the three DRE-related sequences placed upstream of the heterologous TATA promoter, derived from the metallothionein gene basal promoter, are illustrated. These reporter plasmids were transfected into Kc cells, and luciferase activities were determined and expressed as values relative to that with the construct carrying the metallothionein gene basal promoter alone.

As shown in Fig. 11D, strong staining signals with the anti-β-galactosidase antibody were observed in cells posterior to the morphogenetic furrow on compared with the control fly case (Fig. 11C). No significant difference in the lacZ expression pattern in flies with and without DREF overexpression was observed in cells anterior to the furrow, a region where DREF is not overexpressed. Thus, we conclude that DREF can activate the dE2F gene promoter in vivo.

DISCUSSION

Promoters of Drosophila DNA replication-related genes contain common transcriptional regulatory elements such as E2F-binding sites and DRE (20, 23, 25, 29). Our previous studies using a transgenic fly system indicated that both E2F-binding sites and DRE are required for activity of the Drosophila PCNA gene promoter throughout all stages of development (30). dE2F is essential for transcription of Drosophila DNA replication-related genes, since transcripts of the latter are completely
lacking in the dE2F- mutant embryos (18, 19, 21, 22). Cyclin E, which in the form of a complex with cyclin dependent kinase a critical regulator of G1 to S phase transition, activates dE2F in central nervous system cells (18). This regulation appears to be mediated by a homologue of the retinoblastoma protein, RBF (44).

In addition to this physiological regulation of dE2F activity, the level of dE2F can be increased through a transcription step during the onset of proliferation. In mammals, auto-regulation through E2F-binding sites in the E2F gene promoters and several transcription factors such as CCAAT-binding protein, YY-1 and c-Myc, have been suggested to play important roles in transcriptional activation of E2F genes (8, 11, 13). However, in the Drosophila case, little is known about transcriptional regulators. In the present study, we have shown that the DRE/DREF system activates transcription of the dE2F gene. Probably during the onset of proliferation, increased dE2F activity in combination with DREF could coordinate activation of DNA replication-related genes to prepare for S phase.

Adult eyes of transgenic flies overexpressing DREF in the eye imaginal discs shows a severe rough eye phenotype. A half-reduction of the dE2F gene copy number suppresses this rough eye phenotype. However, half-reductions of other DREF-target genes such as those for PCNA, DNA polymerase α, and cyclin A apparently exerted no effect on the rough eye phenotype. Thus, the dE2F gene appears to be one of the most critical target genes of DREF, at least in eye imaginal disc cells.

We have found that overexpression of DREF can stimulate the expression of the lacZ gene that is placed under the control of the dE2F gene promoters in the eye imaginal disc. Since the lacZ transgene is located downstream of two different dE2F gene promoters, one for transcript-a and the other for transcript-b, we do not know which promoter is actually activated by DREF. The present studies indicate that the DRE-related sequence located upstream of the initiation site of transcript-b is required for the promoter activity and specifically bind DREF. Although we also found DRE-related sequences in the upstream region of the initiation site of transcript-a, these were not recognized by DREF on band mobility shift analysis (data not shown). Thus, the overexpression of DREF likely activates the promoter for transcript-b rather than that for transcript-a. It should be noted that the promoter for transcript-b appears to function throughout all stages of development, while that for transcript-a may only act at limited stages during embryogenesis.

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**Transcriptional Regulation of the Drosophila E2F Gene**

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26051