Essential Role of E2F Recognition Sites in Regulation of the Proliferating Cell Nuclear Antigen Gene Promoter during Drosophila Development*

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We have found sequences similar to the transcription factor E2F recognition site within the Drosophila proliferating cell nuclear antigen (PCNA) gene promoter. These sequences are located at positions –43 to –36 (site I) and –56 to –49 (site II) with respect to the cap site. Glutathione S-transferase (GST)-E2F and GST-DP fusion proteins cooperate and bind to the potential E2F sites in the PCNA promoter in vitro. A binding factor(s) to these sequences that has similar binding specificity to that of E2F was detected in nuclear extracts of Drosophila Kc cells. Furthermore, transient expression of E2F in Kc cells activated the PCNA promoter, and the target site for the activation coincided with the E2F sites. These results indicate that the PCNA gene is a likely target gene of E2F. Examination of lacZ expression from PCNA-lacZ fusion genes carrying mutations in either or both of two E2F sites introduced into flies by germ line transformation revealed that site II plays a major role in the PCNA promoter activity during embryogenesis and larval development, although both sites are required for optimal promoter activity. However, for maternal expression in ovaries, either one of the two sites is essentially sufficient to direct optimal promoter activity. These results demonstrate, for the first time, an essential role for E2F sites in regulation of PCNA promoter activity during development of a multicellular organism.

Many lines of evidence have indicated that the expression of genes involved in DNA replication is closely correlated with the proliferation state of cells and repressed in accordance with progression of differentiation in various tissues during development (1, 2). In budding yeast, genes involved in DNA replication contain a common promoter element (MUI cell cycle box, 5′-ACGCCGCT) (3), and the specific transcription factor complex DSC1 (MBF) is required for expression at the G1-S boundary (4, 5).

In mammalian cells, expression of genes involved in DNA replication increases dramatically at late G1 in response to growth stimulation (6, 7). Many of these genes including the proliferating cell nuclear antigen (PCNA) gene contain the transcription factor E2F-binding site (5′-TTTCCGGC) within their promoter regions (8–10) or a first intron (11). Mammalian E2F is a heterogeneous factor representing the combined activity of at least four gene products called E2F-1, E2F-2, E2F-3, and DP-1. E2F-1 and DP-1 associate into stable complexes and activate transcription in a cooperative manner (12, 13). The regulation of E2F function also appears to play an important role during muscle terminal differentiation (14).

In Drosophila, we have isolated genes for PCNA (15) and the DNA polymerase α (16) and found a common regulatory element, DRE (5′-TATCGATA) and a specific DRE-binding factor, DREF. The DRE-DREF system appears to play a key role in the differentiation-coupled repression of cell proliferation during embryogenesis (17). In addition, cDNAs for Drosophila homologs of E2F-1 and DP-1 have been recently cloned (18, 19). These two proteins interact with each other and cooperate to give sequence-specific DNA binding and optimal trans-activation (19). Furthermore, multiple E2F recognition sites have been identified in the promoter of the Drosophila DNA polymerase α gene (18).

To assess the possibility that the Drosophila PCNA gene might have E2F sites, as is the case with mammalian PCNA genes (11), we searched for sequences similar to those in the DNA polymerase α gene and found two such sequences within the PCNA promoter. We have detected a binding factor(s) to these sequences that has similar specificity to that of E2F. Furthermore, expression of E2F in Kc cells activated the PCNA promoter, and the target site for the activation coincided with the E2F sites. Analyses with transgenic flies indicate that the E2F sites are required for PCNA promoter function throughout Drosophila development.

EXPERIMENTAL PROCEDURES

Oligonucleotides

The sequences of double-stranded oligonucleotides containing potential E2F sites or their base-substituted derivatives in the PCNA promoter were defined as follows.

E2F-P gatccCACATCCCTATCCCGCTCATTTAGCCCGCCTGAAAGTa gTGTAGGGAATAGGGCGGACCTACCCTACCTTACGCTCTa ctctag
E2F-PmutI gatccCACATCCCTCGCCTATCCCGCTCATTTAGCCCGCCTGAAAGTa gTGTAGGGAATAGGGCGGAGTAAATCGGGCGGACTTTCAtctag
E2F-PmutII gatccCACATCCCTTcgcGCTATTAGGCCGGTGAAGGAGT gcgGTGTAGGGGAAAGGTTGGGGACTTTCAtctag
E2F-PmutI&II gatccCACATCCCTCgcGCTATTAGGCCGGTGAAGGAGT gcgGTGTAGGGGAAAGGTTGGGGACTTTCAtctag

The sequences of double-stranded oligonucleotides containing E2F sites in the DNA polymerase α promoter were as follows.

Polosite1 attttgaccTGGATTTCCCCTCGGCTAATTAT 9gAGCCAAAAGGGCGTTTATA
Polosite2+3 gatcCCGATGCTTTCCCTGGCAAAATAT 9gAGCCAAAAGGGCGTTTATA

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1 The abbreviations used are: PCNA, proliferating cell nuclear antigen; DRE, Drosophila response element; URE, upstream response element; DREF, Drosophila response element factor; PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase.

http://www.jbc.org/content/270/42/25159.full.pdf
Role of E2F Recognition Sites during Development

The sequences of double-stranded oligonucleotides containing two E2F sites or their base-substituted derivatives in the adenovirus E2 promoter (20) were as follows.

\[ \text{CGCCTGTGGCTTTTCACATCCCTcgCaaGCTCATTTTAGCC; mutI&II, CCTATCCCGCTCATTTctCaaGCCTGAAAGT; mutII, 5'-GGCGATAT-} \]

\[ \text{9} \]

\[ \text{AAGCTCGAGC; mutI, 5'-cleotides used were as follows: CAT-1, 5'-} \]

\[ \text{6-base pair linker sequence (21). DRE-PM contains a 2-base pair sub-} \]

\[ \text{site of the 24-base pair DRE sequence of the PCNA gene promoter and the} \]

\[ \text{AdE2Fmut sites or their base-substituted derivatives in the adenovirus E2 pro-} \]

\[ \text{moter (27). Each nuclear extract was incubated in 15 mg of poly(dI-dC) for 10 min} \]

\[ \text{on ice. The double-stranded oligonucleotide, DRE-P,} \]

\[ \text{contains the luciferase gene under control of the} \]

\[ \text{AdE2FmutW8HS.} \]

\[ \text{Expression of GST Fusion Proteins and Gel Mobility Shift Assay} \]

\[ \text{Expression of GST-E2F and GST-DP fusion proteins was carried out as} \]

\[ \text{described elsewhere (29). Lysates of cells were prepared by sonica-} \]

\[ \text{tion in buffer D containing 0.6 M KCl, 1 mM phenylmethylsulfonyl} \]

\[ \text{fluoride, 1 mg/ml each of pepstatin, leupeptin, and apronitin. Lysin} \]

\[ \text{ses were cleared by centrifugation at 12,000 \times g for} \]

\[ \text{20 min at} \]

\[ \text{5°C for use on gel} \]

\[ \text{for gel mobility shift assay using a 32P-end-labeled AdE2Fwt oligonu-} \]

\[ \text{cleotide (117 pg) as a probe. The gel mobility shift assay was carried} \]

\[ \text{out as described above except that the reaction mixture for the binding} \]

\[ \text{reaction contained 20 ng Hepes (pH 7.5), 120 mM KCl, 10 mM MgCl_2, 1 mM} \]

\[ \text{EGTA, 0.5 mM dithiothreitol, 10% glycerol, 1 mg/ml of sonicated salmon} \]

\[ \text{DNA.} \]

\[ \text{DNA Transfection into Cells, CAT Assay, and Luciferase Assay} \]

\[ \text{Drosophila Kc cells (30) were grown in M3(6B) medium supple-} \]

\[ \text{mented with 2% fetal calf serum (31). Cells were plated at about 5 \times 10^6} \]

\[ \text{cells/60-mm dish for 16 h before DNA transfection. DNA was trans-} \]

\[ \text{fected into cells by the calcium phosphate coprecipitation technique} \]

\[ \text{described elsewhere (32). On day 15, 1 \mu g of DNA plasmid as a report-} \]

\[ \text{er plasmid and 0.1 \mu g of pDHsp70L, an internal control plasmid, were} \]

\[ \text{cotransfected with the indicated amount of the effector plasmid. The} \]

\[ \text{total amount of effector plasmid was kept constant by the addi-} \]

\[ \text{tion of the vector pAcGEM3 (22), and the total amount of DNA was} \]

\[ \text{adjusted to} \]

\[ \text{10 \mu g by the addition of pGEM3. Cells were harvested at} \]

\[ \text{48 h after transfection. Cell extracts were prepared, and CAT activity} \]

\[ \text{was measured as described (33). Radioactivity of acetylated chloro-} \]

\[ \text{phenolic on thin-layer plates was quantified with an imaging analyzer} \]

\[ \text{BAS2000 (Fuji Film).} \]

\[ \text{The luciferase assay was carried out by means of a PicaGene assay} \]

\[ \text{kit (Toyo Inc.) as described previously (9). All assays were performed} \]

\[ \text{within the range of linear relation of the activities to incubation time} \]

\[ \text{and protein amounts. CAT activity was normalized to the luciferase} \]

\[ \text{activity.} \]

\[ \text{Establishment of Transgenic Flies} \]

\[ \text{P-element-mediated germ line transformation was carried out as} \]

\[ \text{described (34, 35). G_4} \]

\[ \text{transforms were selected on the basis of white eye color rescue.} \]

\[ \text{Multiple independent lines were obtained for each of the} \]

\[ \text{various fusion genes. Established transgenic strains and their chro-} \]

\[ \text{mosomal linkages are listed in Table I.} \]

\[ \text{Analysis of Expression Patterns for PCNA-lacZ} \]

\[ \text{Quantitative Measurement of \beta-Galactosidase Activity in Extracts} \]

\[ \text{of 36-Male transgenic flies were crossed with female wild type flies.} \]

\[ \text{Groups of 50–100 individuals each of embryos, larvae, pupae, and adult} \]

\[ \text{flies were homogenized in 500 \mu l of ice-cold assay buffer (50 mM} \]

\[ \text{potassium phosphate, pH 7.5, 1 mM MgCl_2). Homogenates were cen-} \]

\[ \text{trifuged at 10,000 \times g at 4 °C for 5 min. For each assay, 50–200 \mu l} \]

\[ \text{of the supernatant was added to give} \]

\[ \text{1 ml of assay buffer containing 1 mM} \]

\[ \text{chlorophenol red-\beta-D-galactopyranoside substrate (Boehringer Mann-} \]

\[ \text{heim). Reaction incubations were at 37 °C in the dark. Substrate} \]

\[ \text{concentration was measured at 574 nm using a spectrophotometer. The} \]

\[ \beta-\text{galactosidase activity was defined as absorbance units/h/mg of protein.} \]

\[ \text{To correct for endogenous \beta-\text{galactosidase activity, extracts from the} \]

\[ \text{wild type strain were included in each experiment, and this back-} \]

\[ \text{ground reading was subtracted from readings obtained with each transformant} \]

\[ \text{line. Deviation among independent strains was less than 30% (not} \]

\[ \text{shown).} \]

\[ \text{Demonstration of \beta-Galactosidase Activity—\beta-Galactosidase activity} \]

\[ \text{of larval and adult tissues was visualized as described elsewhere (36).} \]

\[ \text{After dissection, tissues were incubated in fixative (12 mM sodium} \]

\[ \text{cocoate buffer, pH 7.3, 26% glutaraldehyde) for 15 min at room} \]

\[ \text{temperature. Treated tissues were then incubated with a staining so-} \]

\[ \text{lution containing 0.2% 5-bromo-4-chloro-3-indolyl-\beta-D-galactoside in} \]

\[ \text{the dark at 37 °C for 5–16 h. For photography, tissues were} \]

\[ \text{immersed in glycerol, mounted on slides, and photographed with an Olympus micro-} \]

\[ \text{scope (BX-50) using Tri-X pan 400 films (Kodak).} \]

\[ \text{RESULTS} \]

\[ \text{Potential E2F Recognition Sequences Located in the Prom-} \]

\[ \text{oter Region of the Drosophila PCNA Gene—Three potential E2F sites have} \]

\[ \text{been identified in the Drosophila DNA polym-} \]
TABLE I
Transformants carrying the lacZ fused to the PCNA gene 5'-flanking sequence

| P-element plasmids       | Strains | Chromosome linkage |
|--------------------------|---------|--------------------|
| p5'-168DPCNAIacZW8HS     | 5A      | II                 |
|                          | 19*     | III                |
|                          | 21      | X                  |
|                          | 73      | II                 |
|                          | 89      | II                 |
|                          | 91      | III                |
| p5'-168E2F mut1DPCNAIacZW8HS | 6       | II                 |
|                          | 18      | III                |
|                          | 25      | II                 |
|                          | 26      | II                 |
|                          | 29      | II                 |
|                          | 40      | III                |
| p5'-168E2F mut1IDPCNAIacZW8HS | 5       | II                 |
|                          | 16      | X                  |
|                          | 107     | II                 |
| p5'-168E2F mut1IDPCNAIacZW8HS | 25      | II                 |
|                          | 34      | X                  |
|                          | 72      | III                |

*A line whose lacZ expression pattern is different from that of other lines carrying the same fusion gene.

Fig. 2. Cooperative binding of E2F and DP to the oligonucleotide AdE2Fwt and competition by wild type and mutant E2F-P oligonucleotides. A, radiolabeled double-stranded AdE2Fwt oligonucleotides were incubated with or without (−, lane e) the indicated amounts of lysates from bacteria carrying p6EX-ZT (lanes a–c), pGST-dE2F (lanes b and d), or pGST-dDP (lanes c and d), individually (lane a) or in combination (lanes b–d). B, radiolabeled double-stranded AdE2Fwt oligonucleotides were incubated with or without (−, lane t) 1 μl each of lysates from bacteria carrying pGST-dE2F or pGST-dDP in the presence of the indicated amounts of competitor oligonucleotides (indicated at the top of each lane). AdE2Fwt, oligonucleotides containing two wild type E2F sites from the adenovirus E2 promoter; AdE2F mut, oligonucleotides containing two mutant E2F sites from the E2 promoter; E2F-P, oligonucleotides containing two wild type E2F sites from the PCNA promoter; E2F-2mut, oligonucleotides having a mutation in the E2F site I of the PCNA promoter; E2F-2mutII, oligonucleotides having a mutation in the E2F site II of the PCNA promoter; E2F-2mutI&II, oligonucleotides having mutations in both E2F sites I and II of the PCNA promoter.

Fig. 1. Nucleotide sequences of potential E2F recognition sites in the Drosophila DNA polymerase α and PCNA genes. A, site I in the DNA polymerase α promoter contains an overlapping pair of E2F recognition sequences as indicated by horizontal lines. Locations of each site relative to the cap site are indicated by numbers with vertical lines. B, constructs of wild type PCNA-lacZ (p5'-168DPCNAIacZW8HS) and PCNA-CAT (p5'-168DPCNAICAT) fusion genes are shown. The vertical lines with horizontal arrows indicate the cap site. The open arrow boxes indicate the 5'-untranslated and coding sequences of the PCNA gene, respectively. The dark stippled boxes indicate the DRE sequence. The shaded and the hatched boxes indicate the lacZ coding and CAT coding sequences, respectively. The oligonucleotide sequences in and around the two E2F sites of wild type and mutant PCNA genes are shown. Nucleotides with substitution for the wild type sequence are shown by lowercase letters. Nucleotide sequences of potential E2F recognition sites I and II are indicated by boxes.

erat the α promoter (Fig. 1A) (18). Site I has been demonstrated to be the most effective for binding of Drosophila E2F (18). A search for E2F sites similar to those of the DNA polymerase α gene revealed two such sequences within the PCNA promoter.

These sequences are located at positions −43 to −36 (site I) and −56 to −49 (site II) relative to the cap site (Fig. 1B). Nucleotide sequences of site I and site II, respectively, match seven out of eight and six out of eight nucleotides of the E2F site I in the DNA polymerase α promoter (18) (Fig. 1).

GST-E2F and GST-DP Fusion Proteins Cooperate and Bind to the Potential E2F Recognition Sequences in the PCNA Promoter—Lysates were prepared from bacteria carrying pGST-E2F or pGST-DP, and gel mobility shift assays were carried out. As shown in Fig. 2A, a DNA-protein complex was detected with the AdE2Fwt oligonucleotide containing the two E2F sites of the adenovirus E2 promoter (20) only when both GST-E2F and GST-DP lysates were mixed. Specificity of binding was evident in competition with wild type and mutant E2F sites from the E2 promoter (Fig. 2B, lanes a–d and q–s). E2F-P oligonucleotide (Fig. 1B) containing the two potential E2F sites of the adenovirus E2 promoter effectively competed for the binding (Fig. 2B, lanes e–g). The oligonucleotide E2F-2mutII carrying mutations in the E2F site II competed for the binding (Fig. 2B, lanes h–j) much less efficiently than the wild type E2F-P (Fig. 2B, lanes k–m). In contrast, the oligonucleotide E2F-2mutII carrying mutations in the site II appears to play a major role in the binding.

The potential E2F sites of the PCNA promoter have high affinity for the complex of GST-E2F and GST-DP fusion proteins, and site II appears to play a major role in the binding.
E2F sites in the DNA polymerase α (Fig. 3A), competed for the binding when added to the reaction in excess of the wild-type E2F site from the adenovirus E2 promoter (20). Figure 3 unrelated sequences of similar sizes such as DRE-P or DRE-PM with 32P-labeled E2F-P was diminished by adding an excess using an E2F-P oligonucleotide as a probe. The band shifted in Fig. 3 in cells, and gel mobility shift assays were carried out. As shown in Fig. 3A, a specific DNA-protein complex could be detected using an E2F-P oligonucleotide as a probe. The band shifted with 32P-labeled E2F-P was diminished by adding an excess amount of unlabeled E2F-P as a competitor but not by adding unrelated sequences of similar size such as DRE-P or DRE-PM (Fig. 3A, lanes a-d and k-q). The oligonucleotide containing the wild-type E2F site from the adenovirus E2 promoter (20) competed for the binding when added to the reaction in excess (Fig. 3A, lanes r-t). Similarly, oligonucleotides containing the E2F sites in the DNA polymerase α promoter (18) competed for the binding (Fig. 3A, lanes e-j). In contrast, the oligonucleotide containing the mutant E2F site from the adenovirus E2 promoter did not compete under the examined conditions (Fig. 3A, lanes u-w). These results indicate that a binding factor(s) to the potential E2F sites in the PCNA promoter has binding specificity indistinguishable from that of Drosophila E2F (18).

As shown in Fig. 3B, the oligonucleotide E2F-PmutI carrying mutations in the E2F site I (Fig. 1B) competed for the binding as effectively as the wild type E2F-P. In contrast, the oligonucleotide E2F-PmutII carrying mutations in the E2F site II (Fig. 1B) only weakly competed for the binding (Fig. 3B, lanes g-k). Therefore, site II appears to play a major role in the binding.

Effects of Mutations in the Potential E2F Sites on PCNA Promoter Activity in Kc Cells—The PCNA promoter carrying mutations in either or both of two E2F sites was placed upstream of the CAT gene in a CAT vector (Fig. 1B). These plasmids were transfected into Kc cells, and CAT expression levels were determined. As shown in Fig. 4, the plasmid carrying mutations in E2F site I showed 41% of CAT expression as compared with that of the original plasmid. Much more extensive reduction of CAT activity was observed with the plasmid p5'-168E2FmutI&II-DPCNACAT carrying mutations in E2F

FIG. 3. Complex formation between E2F-P oligonucleotides and Kc cell nuclear extract and competition by various oligonucleotides. Radiolabeled double-stranded E2F-P oligonucleotides were incubated with Kc cell nuclear extract (2 μg of protein) in the presence or absence (0) of the indicated amounts of competitor oligonucleotides (indicated at the top of each lane). A, E2F-P; oligonucleotides containing two wild type E2F sites from the PCNA promoter; polα wild2.2-3, oligonucleotides containing E2F sites 2 and 3 from the DNA polymerase α promoter; polα site1, oligonucleotides containing the E2F site 1 from the DNA polymerase α promoter; DRE-P, oligonucleotides containing the DRE sequence from the PCNA promoter; DRE-PM, oligonucleotides having a mutation in the DRE sequence; AdE2F wt, oligonucleotides containing two wild type E2F sites from the adenovirus E2 promoter; AdE2F mut, oligonucleotides containing two mutant E2F sites from the E2 promoter; B, oligonucleotides having a mutation in E2F site I of the PCNA promoter (E2F-PmutI) and oligonucleotides having a mutation in the E2F site II of the PCNA promoter (E2F-PmutII).

FIG. 4. Effects of mutations in E2F sites on PCNA promoter activity in Kc cells. One μg each of CAT plasmids harboring wild type or mutant PCNA promoters (indicated at the top of each lane) were cotransfected with 0.1 μg of pDhs70-L plasmid into Kc cells. 48 h after the transfection, cell extracts were prepared to determine the CAT expression levels, normalized to the luciferase activity. Averaged values obtained from two independent dishes with standard deviations are given as CAT activity relative to that of p5'-168DPNCNACAT (−168, lanes a and b). Promoterless CAT (pSKCAT) plasmids were included as controls (lanes k and l). Acetylated forms of [3H]chloramphenicol were undetectable in lanes i–l. Acetylated and nonacetylated forms of [3H]chloramphenicol are marked by Ac and CM, respectively. −168, p5'-168PCNACAT; −168mutI, p5'-168 mutI-DPCNACAT; −168mutII, p5'-168 mutII-DPCNACAT; −168mutI&II, p5'-168 mutI&II-DPCNACAT; −86, p5'-86DPCNACAT; −168 mutI&E2F-P(N)-DPCNACAT; −168 mutII&E2F-P(N)-DPCNACAT; −168 mutI&II&E2F-P(R)-DPCNACAT; p5'-168 mutI&E2F-P(R)DPCNACAT.

FIG. 5. Effect of cotransfecting E2F or DP expression plasmid on the CAT activity directed by the regulatory region of the PCNA gene. 0.5 μg each of plasmid p5'-168DPNCNACAT (upper panel) or p5'-116DPNCNACAT (lower panel) was cotransfected into Kc cells with 0.1 μg of pDhs70-L plasmid and the indicated amounts of ActdE2F (open circles), pAdE2F 1WT (closed circles) or Act-dDP (closed squares). 48 h after the transfection, cell extracts were prepared to determine the CAT expression levels, normalized to the luciferase activity and plotted against activity in the absence of the effector plasmid. Averaged values obtained from three independent transfections are shown.
and wild-type PCNA-CAT forms of [14C]chloramphenicol were undetectable in lanes m–r. These results indicate that E2F site II plays a major role, and site I might play an additional role in regulation of the PCNA promoter activity.

These E2F sites are essential but not sufficient for the promoter activity, since deletion up to position −86 completely abolished the promoter activity, even when the two E2F sites were kept intact (Fig. 4, lanes i and j). In addition, insertion of the E2F-P downstream of the CAT gene of the plasmid p5'-168E2FmutI&II DPNCNACAT did not enhance CAT expression (Fig. 4, lanes m–r), indicating the importance of the position of E2F sites for activation of transcription.

Activation of PCNA Promoter-directed CAT Expression by E2F—To determine whether the PCNA promoter can be activated by E2F, a cotransfection assay using Kc cells was carried out. Expression of the E2F protein activated PCNA promoter-directed CAT expression 2-fold (Fig. 5, upper panel). However, expression of the DP protein did not affect CAT expression. When plasmid p5'-116DPNCNACAT, carrying the region from −116 to +23 of the PCNA gene linked to the CAT-coding region, was used as the reporter plasmid, more extensive activation of CAT expression was observed with E2F-expressing plasmids (Fig. 5, lower panel). Here too, the DP-expressing plasmid had no effect on CAT expression. In addition, when the DP-expressing plasmid was cotransfected with the reporter plasmid and the E2F-expressing plasmid, no further activation of CAT expression was observed (not shown). These results indicate that the E2F protein can activate the PCNA promoter (as is the case with the DNA polymerase α promoter (18)), and the level of the E2F protein but not that of the DP protein appears to be limiting for the activation in Kc cells.

Mapping of the Target Region for Activation by E2F Protein—A set of 5'-deletion derivatives of the plasmid p5'-168DPNCNACAT were cotransfected with the E2F-expressing plasmid. Deletions toward position −116 caused a gradual decrease of CAT expression and a progressive increase of activation by E2F (Fig. 6A). A further deletion to position −86 completely abolished the CAT expression, and accordingly, the stimulation by E2F was no longer detectable.

To examine the responsibility of E2F sites for the activation by E2F, base substitution derivatives of p5'-168DPNCNACAT were cotransfected with the E2F-expressing plasmid. As shown in Fig. 6B, the E2F-expressing plasmid still activated CAT expression from plasmids carrying mutations in either of two E2F sites. However, mutations in both sites completely abolished the response to E2F expression (Fig. 6B, lanes m–p). Therefore, at least one of two E2F sites is required for the E2F protein to activate the PCNA promoter.

Role of E2F Sites in Function of the PCNA Promoter in Living Flies—Although the results of CAT transient expression assay in Kc cells clearly demonstrate the essential role of E2F sites in the PCNA promoter activity, these observations have to be confirmed in living flies, and transgenic Drosophila provides an appropriate system to characterize transcriptional regulatory elements in vivo. We have established transgenic flies carrying PCNA (−168 to +137) and lacZ fusion genes (Fig. 1B) (22). Male transgenic flies were crossed with wild type females to examine zygotic expression of the lacZ. Expression of lacZ was found to be high in embryos, first and second instar larvae, and adult females and low at other stages of development (Fig. 7, top panel) (37). To examine the role of E2F sites in the PCNA promoter activity during Drosophila development, we generated PCNA-lacZ fusion genes carrying base substitutions in either or both
of two E2F sites. These fusion genes were then introduced into flies by germ line transformation. Activities of modified promoters were then monitored by the quantitative \( \beta \)-galactosidase activity in the salivary glands, the brain lobes, and the imaginal discs of third instar larvae and in the ovaries of adult females. Salivary glands (panels A–D), brain lobes (panels F–J), and leg discs (panels K–O) were dissected from the third instar larvae of male transgenic flies carrying the fusion gene (indicated at the left side of each panel) \( \times \) wild type females. Ovaries (lanes P–T) were dissected from 3-day-old adult females carrying the transgenes indicated at the left side of each panel. They were then subjected to demonstration of \( \beta \)-galactosidase activity. Tissues from Canton S larvae and adult females carrying no transgene were processed in the same way as controls (panels E, J, O, and T). 

In mammalian cells, a group of genes that are commonly regulated in late G1 of the growth response and that encode proteins important for DNA replication appear to be regulated by E2F (8). In Drosophila, multiple E2F sites have been identified in the gene for the 180-kDa catalytic subunit of the DNA polymerase \( \alpha \) (18). In the present study, we have identified two E2F recognition sites in the PCNA promoter. It is thus clearly of interest to identify the presence of E2F site(s) in the promoter regions of other DNA replication-related genes in Drosophila.

Molecular cloning of two other Drosophila genes involved in DNA replication has been so far reported. One is the gene for the 73-kDa regulatory subunit of the DNA polymerase \( \alpha \) (38), and the other is that for the 50-kDa subunit of the DNA primase (39). The former gene contains three potential E2F sites in its 5′-flanking region. Two of them (5′-TTTTCGCAG and 5′-CTTCGGG) match seven out of eight and six out of eight nucleotides of the binding consensus (5′-TTTTCGGG) for mammalian E2F, respectively. The other site (5′-TTACCCGG) matches seven out of eight nucleotides of the E2F recognition site I of the DNA polymerase \( \alpha \) 180-kDa subunit gene. The
50-kDa primase gene also contains a potential E2F site in its 5'-untranslated region. This site (5'-ATTCCCAG) perfectly matches the nucleotide sequence of the E2F site 3 of the DNA polymerase α gene. Although promoter sequence information is not available for other Drosophila genes involved in DNA replication, we predict that they very likely contain E2F sites, as is the case with mammalian DNA replication-related genes.

In our previous studies of Drosophila genes for PCNA and DNA polymerase α, we found a common regulatory element, DRE (21), which therefore appeared to be an important element for at least these two genes. DRE is essential for the function of the PCNA promoter both in embryos and in larvae (26). Since DRE was found to be by itself not sufficient to activate the PCNA promoter during larval stages, we searched for another regulatory element and found an upstream regulatory element (URE) located in the region from nucleotide position −168 to −119 (to be published elsewhere). Since the URE sequence alone was also not sufficient to activate the PCNA promoter in larvae, both URE and DRE appear to be required to activate the promoter during larval stages.

In the present study, we have identified two E2F sites in the region downstream of DRE of the PCNA gene. Analyses with transgenic flies demonstrated that these sites are essential for PCNA promoter activity throughout development. However, E2F sites alone proved to be insufficient for PCNA gene promoter activity during embryonic and larval stages, since deletion of the upstream region containing URE and DRE sequences completely abolished the promoter activity during these stages (to be published elsewhere). Thus, URE, DRE, and E2F sites likely cooperate to direct optimal PCNA promoter activity during these stages.

A number of studies have been conducted to explore the regulation of E2F during the cell cycle. Critical roles of E2F sites for regulated expression in late G1 have been demonstrated with the mammalian genes for DHFR (10) and PCNA (11). However, such observations with cultured cell systems have to be confirmed in living organisms, and in this sense transgenic Drosophila provides an appropriate system to characterize E2F sites in vivo. The present study with transgenic flies provides the first evidence for an essential role of E2F sites in regulation of the promoter activity of genes involved in DNA replication during development of a living organism.

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