Repression of the *Drosophila* Proliferating-Cell Nuclear Antigen Gene Promoter by zerknüllt Protein

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A 631-bp fragment containing the 5'-flanking region of the *Drosophila melanogaster* proliferating-cell nuclear antigen (PCNA) gene was placed upstream of the chloramphenicol acetyltransferase (CAT) gene of a CAT vector. A transient expression assay of CAT activity in *Drosophila* Kc cells transfected with this plasmid and a set of 5'-deletion derivatives revealed that the promoter function resided within a 192-bp region (−168 to +24 with respect to the transcription initiation site). Cotransfection with a zerknüllt (*zen*)-expressing plasmid specifically repressed CAT expression. However, cotransfection with expression plasmids for a nonfunctional *zen* mutation, even-skipped, or bicoid showed no significant effect on CAT expression. RNase protection analysis revealed that the repression by *zen* was at the transcription step. The target sequence of *zen* was mapped within the 34-bp region (−119 to −86) of the PCNA gene promoter, even though it lacked *zen* protein-binding sites. Transgenic flies carrying the PCNA gene regulatory region (−607 to +137 or −168 to +137) fused with *lacZ* were established. When these flies were crossed with the *zen* mutant, ectopic expression of *lacZ* was observed in the dorsal region of gastrulating embryos carrying the transgene with either construct. These results indicate that *zen* indirectly represses PCNA gene expression, probably by regulating the expression of some transcription factor(s) that binds to the PCNA gene promoter.

The proliferating-cell nuclear antigen (PCNA) (35), also known as cyclin (8), is a nuclear protein, the expression of which correlates with the proliferating state of the cells (33, 48). PCNA is an auxiliary protein for DNA polymerase δ (9, 42) and is one of the essential factors for synthesis of the leading strand in the replication of simian virus 40 DNA (40, 41). This protein is also important for cellular DNA synthesis and cell cycle progression (27). The cDNAs and genes for mammalian (1, 34, 50), plant (47), *Drosophila* (52), and yeast (2) PCNA have been cloned. Nucleotide sequence analysis revealed that the amino acid sequence of PCNA has been highly conserved during evolution (2, 47, 50); hence, PCNA probably has a vital role in the maintenance of life.

The presence of clusters of 10-bp sequences similar to the binding consensus for *Drosophila* homeodomain proteins (15, 25, 37) was noted in the region from nucleotide positions −165 to −357 of the *Drosophila* PCNA gene (52). DNase I footprint analysis revealed that the homeodomain proteins encoded by a pair rule gene, even-skipped (*eve*), and a dorsal-ventral gene, zerknüllt (*zen*), can specifically bind to these sequences in vitro (52). These observations suggested that expression of the PCNA gene is under the control of genes coding for homeodomain proteins. It may be reasonable to assume that *zen* represses the expression of PCNA, since *zen* directs differentiation of cells that are mitotically inactive (17, 21).

In this study, we dissected the 5'-flanking region of the *Drosophila* PCNA gene and located the region required for promoter activity functioning in cultured cells and living organisms. Han et al. reported that *zen* is a potent transcriptional activator (22); however, the *zen*-producing plasmid specifically repressed the chloramphenicol acetyltransferase (CAT) expression directed by the regulatory region of the PCNA gene. Analysis of the target sequence for this repres-

sion indicated an indirect role of the *zen* protein in the regulation of PCNA gene expression. In vivo studies with transgenic flies corroborate this conclusion. The biological significance of repression of PCNA gene expression by *zen* is discussed.

MATERIALS AND METHODS

Plasmid constructions. A 631-bp DNA fragment containing the −607 to +24 region of the PCNA gene was isolated from a *Drosophila* PCNA genomic DNA clone, pUC19DgPCNA01 (52), by digestion with *Eco*RI, blunt ending by Klenow fragment, digestion with *Sac*II, and insertion into the *Sma*I and *Sac*II sites of plasmid pSKCAT. pSKCAT carried the polylinker region of Bluescript SK(−) in front of the CAT-coding region, splicing signals of the simian virus 40 early gene, and poly(A) addition signal of simian virus 40 early gene. The resultant plasmid was designated p5'−607DPC NACAT. A 428-bp fragment was isolated from pUC19D gPCNA01 by digestion with *Bam*HI, blunt ending by Klenow fragment, digestion with *Sac*II, and insertion into the *Sma*I and *Sac*II sites of pSKCAT to obtain plasmid p5'−404DPCNACAT. This plasmid was then digested with *Sal*I, and a set of 5'-deletion mutants was constructed by digestion with Bal31 nuclease and introduction of the *Sal*I linker at the deletion breakpoints. The deletion breakpoint of each deletion derivative was determined by the dideoxy sequencing method (45), using either a synthetic 17-mer (5'-GGTGGTATATCCAGTGA-3') that hybridized to the CAT-coding region or a 17-mer (5'-TCGATAGCAGGCAG CGA-3') that hybridized to nucleotide positions −95 to −111 of the PCNA gene. The breakpoints of the deletion derivatives are summarized in Fig. 1.

Plasmid p5'−404DPCNACAT was digested with *Cla*I, and a 307-bp fragment was removed. The remaining portion of the plasmid was religated to obtain plasmid p5'−97DPCNA CAT. This plasmid was digested with *Pvu*II and *Cla*I, and
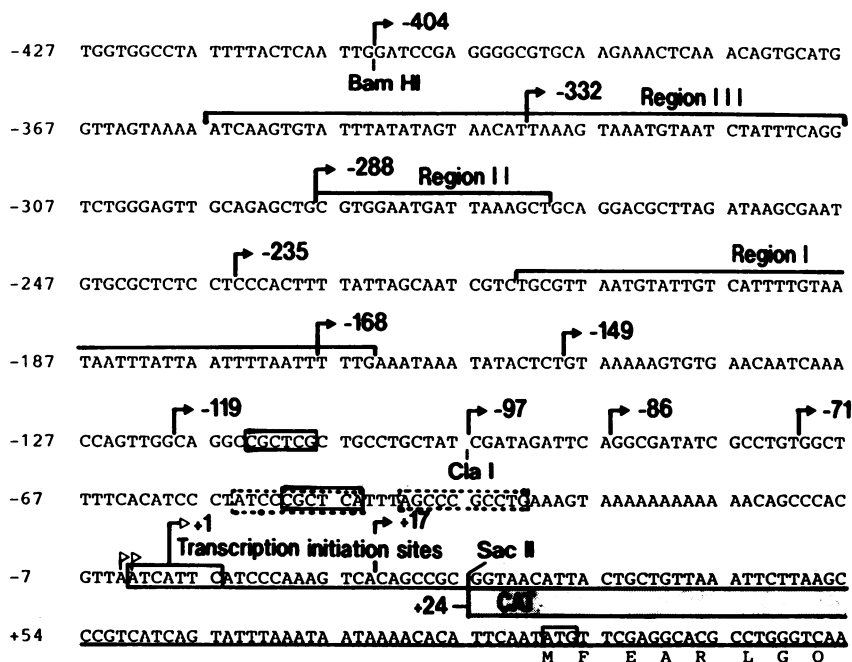
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                                         ┌─► -404
    -427   TGGTGGCCTA TTTTACTCAA TTCGATCCGA GGGGCGTGCA AGAAACTCAA ACAGTGCATG
                                       Bam HI              ┌─► -332   Region I I I
    -367   GTTAGTAAAA ATCAAGTGTA TTTATATAGT AACATTAAAG TAAATGTAAT CTATTTCAGG

                           ┌─► -288   Region I I
    -307   TCTGGGAGTT GCAGAGCTGC GTGGAATGAT TAAAGCTGCA GGACGCTTAG ATAAGCGAAT

                   ┌─► -235                                        Region I
    -247   GTGCGCTCTC CTCCCACTTT TATTAGCAAT CGTCTGCGTT AATGTATTGT CATTTTGTAA

                                 ┌─► -168              ┌─► -149
    -187   TAATTTATTA ATTTTAATTT TTGAAATAAA TATACTCTGT AAAAAGTGTG AACAATCAAA

             ┌─► -119                       ┌─► -97     ┌─► -86           ┌─► -71
    -127   CCAGTTGGCA GGCCGCTCGC TGCCTGCTAT CGATAGATTC AGGCGATATC GCCTGTGGCT
                                            Cla I
     -67   TTTCACATCC CTATCCCGCT CATTAGCCC GCCTGAAAGT AAAAAAAAAA AACAGCCCAC
                     ┌─►+1              ┌─► +17
                  PP │Transcription initiation sites   Sac I
      -7   GTTAATCATT CATCCCAAAG TCACAGCCGC GGTAACATTA CTGCTGTTAA ATTCTTAAGC
                               +24 ─│ CAT
     +54   CCGTCATCAG TATTTAAATA ATAAAACACA TTCAATATGT TCGAGGCACG CCTGGGTCAA
                                                        M  F  E  A  R  L  G  Q
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FIG. 1. Nucleotide sequence of the 5' upstream region and a part of exon 1 of the *Drosophila* PCNA gene. Nucleotide +1 denotes the most proximal major cap site; residues preceding it are indicated by negative numbers. Three cap sites determined by primer extension analysis (52) are indicated by vertical lines with open arrowheads. The sequence that matches the cap site consensus of insect mRNAs (26) is boxed. A series of 5'-deletion mutants was constructed, and their 5' ends are indicated by numbered vertical lines with closed arrowheads. The regions (I to III) interacting with homeodomain proteins in vitro (52) are indicated by bars above the sequence. The position of the sequence (+24) that was linked to the CAT vector DNA (CAT) is also shown. The untranslated region of the mRNA is indicated by a solid line below the nucleotides. The protein-coding region starting with the ATG codon is indicated by the amino acid single-letter code. The 10-nucleotide sequence similar to that of the mammalian transcription factor Sp1-binding consensus is indicated by the dotted box. The hexanucleotide sequence that matches the *Drosophila* zeste protein-binding consensus is enclosed in a solid box.

then a 279-bp fragment was isolated. This fragment was inserted into *Sal*I and *Sma*I sites of Bluescript SK(−) to obtain plasmid pBCP, which was used to prepare the RNA probe for RNase protection analysis.

The expression plasmid pAct5C-zen (22) contains full-length *zen* cDNA placed under the control of the *Drosophila* actin 5C promoter (−2500 to +88) (6). Similarly, the expression plasmids pAct5C-eve (22) and pPAcbcdEE (18) contain full-length *eve* and *bicoid* cDNAs, respectively. Efficient expression of these proteins in transfected *Drosophila* embryonic cells has been demonstrated with use of specific antibodies to these proteins (18, 22). The expression plasmid pAct5C-zen-Δ1 (22) contains an internal deletion from amino acids 137 to 236 of the *zen* protein, and plasmid pAct5 C-zen-Δ2 (22) contains a deletion from amino acid 235 to the C-terminal end of the *zen* protein. Plasmid pAct5C-z2 is an expression plasmid for the *zen*-related gene z2 (22). Plasmid 5k'-TATA-CAT (22) contains five tandem head-to-tail repeats of fragment k' (−852 to −949 of the engrailed gene) fused to a metallothionein basal promoter. Plasmid pAc-GEM3 contains the actin 5C promoter (−2600 to +76) within the polylinker region of plasmid pGEM3.

A 800-bp DNA fragment containing the termination codon (TAA) and poly(A) addition signal (AATAAA) of the *Drosophila hsp70* gene was isolated from plasmid pryHS (24) by digestion with *Sal*I and *Bgl*II and inserted between the *Xho*I site and blunt-ended *Pst*I site of plasmid pW8 (30). The resultant plasmid was designated pW8HS. A 744-bp DNA fragment (−607 to +137 of the PCNA gene) was isolated from plasmid pUC19DgPCNA01 by digestion with *Bgl*II and

*Eco*RI, blunt ended by Klenow fragment, and then inserted into the unique *Sma*I site of plasmid pMC1871, a β-galactosidase (*lacZ*) gene fusion vector. A DNA fragment containing the *lacZ* gene fused with the PCNA gene in frame was isolated from this plasmid by digestion with *Sal*I, blunt ended by Klenow fragment, and inserted into the blunt-ended *Xba*I site of plasmid pW8HS. The resultant plasmid was designated p5'−607DPCNAlacZW8HS. Plasmid p5'−168DPCNAlacZW8HS was obtained by replacing the DNA fragment between the *Sal*I and *Sac*II sites of plasmid p5'−607DPCNAlacZW8HS with the DNA fragment isolated from p5'−168DPCNACAT by digestion with *Sal*I and *Sac*II.

All plasmids were propagated in *Escherichia coli* HB101 and isolated by standard procedures (44). Isolated plasmid DNA was further purified through two cycles of ethidium bromide-CsCl density gradient centrifugation.

Cell culture, DNA transfection, and CAT assay. *Drosophila* Kc cells (20) were grown in M3(BF) medium (14) supplemented with 2% fetal calf serum. Cells were plated at about 4 × 10⁶ cells per 60-mm dish at 16 h before DNA transfection. DNA was transfected into cells by the calcium phosphate coprecipitation technique described elsewhere (16). Unless otherwise specified, each transfection contained 1 μg of promoter-CAT plasmids, 6 μg of expression plasmid, 3 μg of pAcGEM3, and 7.5 μg of pGEM3 per dish. Cells were harvested at 48 h after transfection. Cell extracts were prepared, and CAT activity was measured as described previously (51). To quantify CAT activity, radioactive spots corresponding to acetylated forms of chloramphenicol were taken from the thin-layer plates, and radioactivity was

measured in a toluene-based scintillation cocktail, using a scintillation counter. CAT activities were normalized to protein amounts, which were determined by Bio-Rad protein assay (51).

RNA preparation and RNase protection analysis. Total cellular RNA was extracted from DNA-transfected Kc cells by the guanidinium HCl-CsCl gradient method (11). Isolated RNA was treated with DNase I in the presence of 100 U of human placental RNase inhibitor to remove contaminating plasmid DNA (44).

RNase protection assays were carried out as described elsewhere (44). Plasmid pBCP was linearized with SalI and transcribed with T3 RNA polymerase in the presence of [α-^{32}P]UTP to yield an RNA probe of 352 nucleotides. Ten micrograms of Kc cell total RNA or yeast tRNA was mixed with 5×10^5 cpm of RNA probe in 30 μl of solution containing 80% formamide, 40 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES; pH 6.4), 0.4 M NaCl, and 1 mM EDTA. After the solution was heated at 85°C for 10 min, hybridizations were carried out at 42°C for 16 h. RNase digestions were performed by incubation at 30°C for 30 min after addition of 300 μl of solution containing 0.3 M NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 2 μg of RNase T$_1$ per ml, and 40 μg of RNase A per ml. RNases were inactivated by proteinase K digestion followed by phenol-chloroform extraction. The protected fragments were applied to a 6% polyacrylamide gel containing 8 M urea. ^{32}P-labeled pUC19 MspI fragments were used as size markers.

Germ line transformation and analysis of expression patterns. P-element-mediated transformation and establishment of homozygous transformant stocks were done as described previously (43, 46). Twenty and ten independent transformant lines were established for p5'$-$607DPCNAlacZW8HS and p5'$-$168DPCNAlacZW8HS, respectively. Male transgenic flies were crossed with female wild-type flies to observe the zygotic expression of lacZ in early embryos (0 to 5 h old). To test the expression of the PCNA-lacZ fusion gene in embryos carrying the mutation of zen (a third chromosomal mutation), males heterozygous for the zen mutation (zen^{w36}/TM3) with the transgene on the X chromosome were crossed with zen^{w36} (32) heterozygous females. Homozygous embryos were identified by characteristic morphological changes in embryogenesis.

Expression patterns of lacZ and endogenous PCNA were analyzed by immunostaining as described previously (31). Embryos (0 to 5 h old) were collected, dechorionated, fixed, devitellinized, and then blocked with Tris-buffered saline (TBS; 50 mM Tris-HCl [pH 8.3], 150 mM NaCl) containing 10% goat serum and 0.15% Triton X-100 (TBT). Incubation with the primary antibody was carried out in the same solution for 16 h at 4°C. The embryos were washed extensively in TBS containing 0.3% Triton X-100, reblocked with TBT, and incubated with the secondary antibody for 16 h at 4°C. After extensive washing with TBS containing 0.3% Triton X-100, color was developed in a solution containing 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5 mM MgCl$_2$, 0.34 mg of nitroblue tetrazolium salt per ml, and 0.175 mg of 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt per ml. A polyclonal rabbit anti-Drosophila PCNA (38) (used at a dilution of 1:1,000) and an alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG; preabsorbed against fixed 0- to 16-h-old embryos and used at a dilution of 1:2,000; Promega Biotec) were used as primary and secondary antibodies for the detection of PCNA. A mouse anti-β-galactosidase (lacZ) monoclonal antibody (preabsorbed against fixed 0- to 16-h-old embryos and used at a dilution of

1:1,000; Promega Biotec) and an alkaline phosphatase-conjugated goat anti-mouse IgG (preabsorbed against fixed 0- to 16-h-old embryos and used at a dilution of 1:2,000; Promega Biotec) were used as primary and secondary antibodies for the detection of lacZ. Preparations were observed with a microscope (Nikon FXA-A1) and photographed on Tri-X Pan 400 film (Kodak).

RESULTS

Repression of PCNA promoter-directed CAT expression by the zen protein. Clustered sequences similar to the binding sites for Drosophila homeodomain proteins (15, 28, 37) were noted in the 5'-flanking region of the Drosophila PCNA gene. DNase I footprint analysis revealed that the products of the eve and zen genes expressed in E. coli specifically bound to these regions (52). The three binding regions for homeodomain proteins are shown in Fig. 1.

To determine whether a homeodomain protein(s) can affect transcription of the PCNA gene, a cotransfection assay using cultured Drosophila embryonic Kc cells was carried out. Plasmid p5'$-$607DPCNACAT, carrying the 5' upstream region ($-$607 to $+$24) of the PCNA gene linked to the CAT-coding region, was used as the reporter plasmid. The expression plasmid for zen, pAct5C-zen (22), that for eve, pAct5C-eve (22), and that for bicoid, pPAcbcdEE (18), were used as effector plasmids. Expression of each of these proteins is directed by the Drosophila actin 5C promoter (6), which is highly active in Drosophila cells. Expression of the zen protein repressed PCNA promoter-directed CAT expression by 70 to 80%. The expression of the eve protein did not affect CAT expression, and expression of the bicoid protein only marginally repressed CAT expression (Fig. 2).

As shown in Fig. 3, the zen expression plasmid activated extensively the CAT expression of plasmid 5k'-TATA-CAT (22), which carries 30 tandem repeats of the consensus homeodomain protein-binding sequence. At the same concentration, it repressed CAT expression directed by the PCNA promoter. Thus, it is not likely that the repression of the PCNA-CAT plasmid is caused by a general squelching phenomenon due to high expression of the zen protein. Furthermore, an internal deletion (zen-Δ1) that lacked 99 amino acids, including the carboxyl-terminal 13 amino acids of the homeodomain (22), eliminated the repression of CAT expression from p5'$-$607DPCNACAT, as did a mutation (zen-Δ2) that deleted 119 amino acids from the carboxyl terminus (22) (Fig. 3, lanes e to h). These mutants did not activate 5k'-TATA-CAT, as noted previously (22) (Fig. 3, lanes m to p). The results suggest that the intact zen protein encoded by the expression plasmid pAct5C-zen is responsible for the repression.

The effect of the zen-related gene z2 (22) on the expression of p5'$-$607DPCNACAT was also examined (Fig. 4). The z2 expression plasmid extensively repressed the CAT expression of p5'$-$607DPCNACAT and strongly activated the expression of 5k'-TATA-CAT.

Since the synergistic effect of multiple homeodomain proteins on target promoters containing homeodomain protein-binding sites had been reported (22, 28), plasmid p5'$-$607DPCNACAT was cotransfected with various combinations of homeodomain protein-producing plasmids. As shown in Fig. 5, repression of CAT expression was observed only when the zen-producing plasmid was included in the transfection; no apparent synergistic effect by other homeodomain proteins was observed.

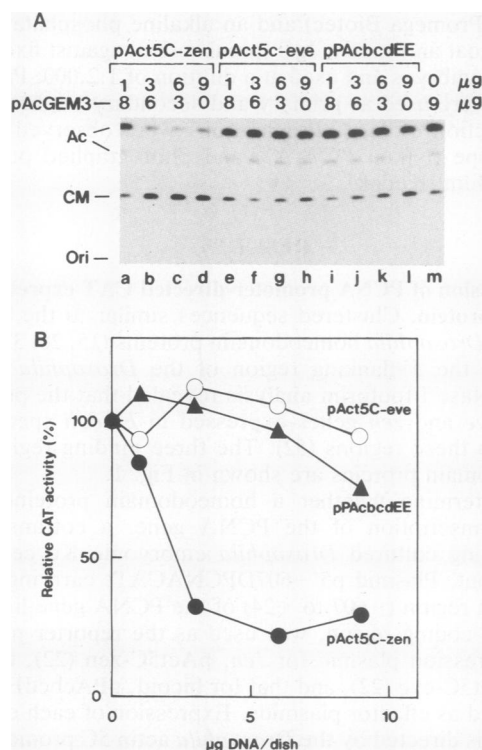**The zen protein represses transcription from the PCNA**

FIG. 2. Effects of cotransfecting homeodomain protein-producing plasmids on CAT expression directed by the regulatory region of the *Drosophila* PCNA gene. (A) One microgram of p5′−607DPCNA CAT DNA and the indicated amounts of pAct5C-zen, pAct5C-eve, or pPAcbcdEE were transfected into Kc cells. The indicated amount of pAcGEM3 DNA and 7.5 μg of pGEM3 DNA were also included to maintain the amount of total DNA at a constant level. At 48 h after transfection, cell extracts were prepared to measure CAT activity. Abbreviations in this and succeeding figures: Ac and CM, acetylated and nonacetylated forms of [14C]chloramphenicol; Ori, origin. (B) The radioactivities of spots corresponding to acetylated [14C]chloramphenicol on thin-layer chromatographic plates shown in panel A were quantified and plotted against activity in the absence of expression plasmid (panel A, lane m). The 100% value was 101,968 cpm.

promoter. We performed an RNase protection analysis to determine whether the proper transcription initiation sites were utilized in the transient expression assay and whether the change in CAT activity observed upon transfection of the zen-producing plasmid was due to a change in mRNA levels. Total RNA was isolated from Kc cells at 48 h after transfection and hybridized to an excess amount of uniformly labeled radioactive antisense RNA probe that was complementary to the region surrounding the previously determined PCNA transcription initiation sites (52) (Fig. 6A). After digestion with RNases A and T₁, protected fragments of nearly the appropriate size, as predicted from the transcription initiation sites determined by the primer extension method, were detected (Fig. 6). The intensity of each radioactive band shown in Fig. 6 was quantified by densitometry. Almost equal amounts of protected fragments were detected with use of RNAs from Kc cells transfected with p5′−607DPC NACAT in combination with either pAcGEM3 (control), pAct5C-eve (*eve* expressing), or pPAcbcdEE (bicoid expressing) (Fig. 6B, lanes d, f, and g). In contrast, cotransfection of this CAT plasmid with the zen-expressing plasmid
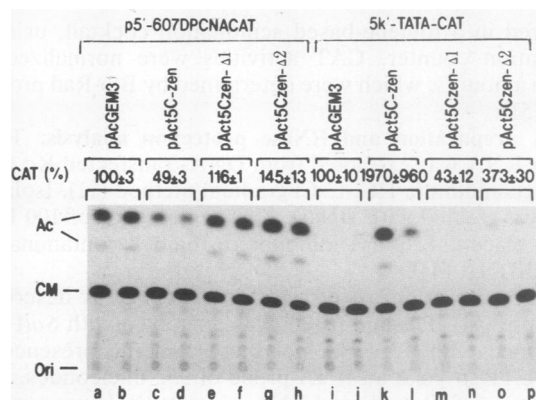

FIG. 3. Effects of deletion mutants of zen on CAT expression from p5′−607DPCNACAT or 5k′-TATA-CAT. Two micrograms of each plasmid was cotransfected into Kc cells with 3 μg of each of the other plasmids indicated above the lanes. At 48 h after transfection, cell extracts were prepared to measure CAT activity. The sets of two adjacent lanes represent duplicate independent transfections. CAT activities (averages ± standard deviations) relative to that of either p5′−607DPCNACAT without expression plasmid (lanes a and b; average 100% value was 39,822 cpm) or 5k′-TATA-CAT without expression plasmid (lanes i and j; average 100% value was 1,776 cpm) are shown.

(pAct5C-zen) resulted in an 82% reduction of the protected fragments (Fig. 6B, lane e). The extent of repression was almost the same as that determined by measurement of CAT enzyme activity (Fig. 2). These results indicate that the zen protein represses transcription from the PCNA promoter.

Mapping of the minimum promoter and the target region of the zen protein. We constructed 5′-end deletion derivatives of plasmid p5′−607DPCNACAT (Fig. 1) and measured the CAT expression level of each construct 48 h after transfection into Kc cells (Fig. 7; quantitative data from four
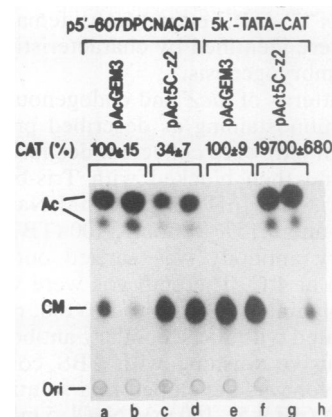

FIG. 4. Effects of the zen-related gene z2 on CAT expression from p5′−607DPCNACAT or 5k′-TATA-CAT. Two micrograms of each plasmid was cotransfected into Kc cells with 3 μg of each of the other plasmids indicated above the lanes. At 48 h after transfection, cell extracts were prepared to measure CAT activity. The sets of two adjacent lanes represent duplicate independent transfections. CAT activities (averages ± standard deviations) relative to that of either p5′−607DPCNACAT without expression plasmid (lanes a and b; average 100% value was 97,811 cpm) or 5k′-TATA-CAT without expression plasmid (lanes e and f; average 100% value was 596 cpm) are shown.
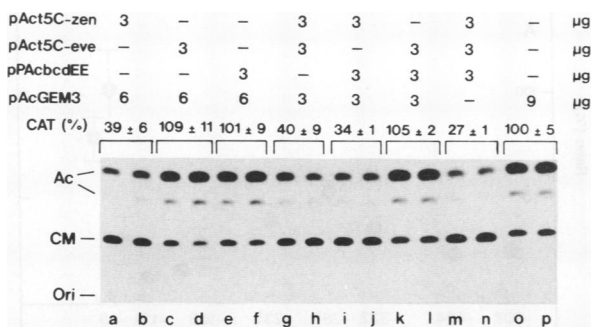
FIG. 5. Effects of cotransfecting various combinations of home-odomain protein-producing plasmids on CAT expression directed by the regulatory region of the *Drosophila* PCNA gene. One microgram of p5'−607DPCNACAT DNA and the indicated amounts of home-odomain protein-producing plasmids and/or control plasmid pAc GEM3 were transfected into Kc cells. At 48 h after transfection, cell extracts were prepared to measure CAT activity. The sets of two adjacent lanes represent duplicate independent transfections. CAT activities (averages ± standard deviations) relative to that of p5'−607DPCNACAT without expression plasmid are shown. The average 100% value was 93,704 cpm.

FIG. 6. RNase protection analysis. (A) Probe used. Plasmid pBCP was linearized with *Sal*I, and T3 RNA polymerase was used to synthesize an RNA probe of 352 nucleotides (nt). The expected protected fragments were about 188 bp. (B) RNase protection analysis of RNA extracted from Kc cells transfected with 6 μg each of either pAcGEM3 (lane d), pAct5C-zen (lane e), pAct5C-eve (lane f), or pPAcbcdEE (lane g), performed as described in Materials and Methods. Similar amounts of total RNA were recovered from cells cotransfected with each expression plasmid. Other lanes: b, undi-gested probe; c, RNase treatment in the presence of yeast tRNA; a and h, DNA size markers. The arrows indicate RNase-protected fragments.

independent experiments are summarized in Fig. 9B). The deletions up to nucleotide position −168 caused no signifi-cant change in CAT expression. Several further deletions toward position −71 caused a gradual but extensive decrease of CAT expression. Thus, the 5' border of the minimum promoter of the PCNA gene resides in the region between −168 and −149.

The target region of the *zen* protein was determined by cotransfecting either of the 5'-deletion derivatives and the *zen*-expressing plasmid. The results of one experiment are shown in Fig. 8, and average values from four independent experiments are shown in Fig. 9B. Ratios of CAT expression with and without the *zen*-expressing plasmid are shown in Fig. 9A. Deletion from positions −404 to −119, which removed the homeodomain protein-binding regions (Fig. 1), caused only a slight fluctuation in the ratio of CAT expres-sion with and without the *zen*-expressing plasmid (Fig. 9). When the deletion was extended from −119 to −97, the response to *zen* expression began to decrease, and further deletion to position −86 abolished the response to *zen* expression. The results indicate that the *zen* protein can exert repressive effects through the 34-bp region between −119 and −86 that resides in the distal portion of the promoter region.

In vivo analysis with transgenic flies. Transgenic flies carrying *lacZ* fused with the PCNA gene regulatory region were established. We transformed flies with two different constructs, p5'−607DPCNAlacZW8HS and p5'-168DPCN AlacZW8HS. Detailed analysis of *lacZ* expression and endogenous PCNA expression patterns during embryogene-sis will be published elsewhere. In this study, we compared the spatial patterns of *lacZ* and PCNA expression in wild-type embryos and embryos with the homozygous *zen* muta-tion. If *zen* represses PCNA gene expression in the embryo, the ectopic expression of PCNA would be observed in the dorsal region of the homozygous *zen* mutant embryo, the site where *zen* functions in the wild-type embryo. The difference in expression of PCNA between the wild-type and *zen* mutant embryos was most evident at the beginning of stage 10, which was characterized by the appearance of the
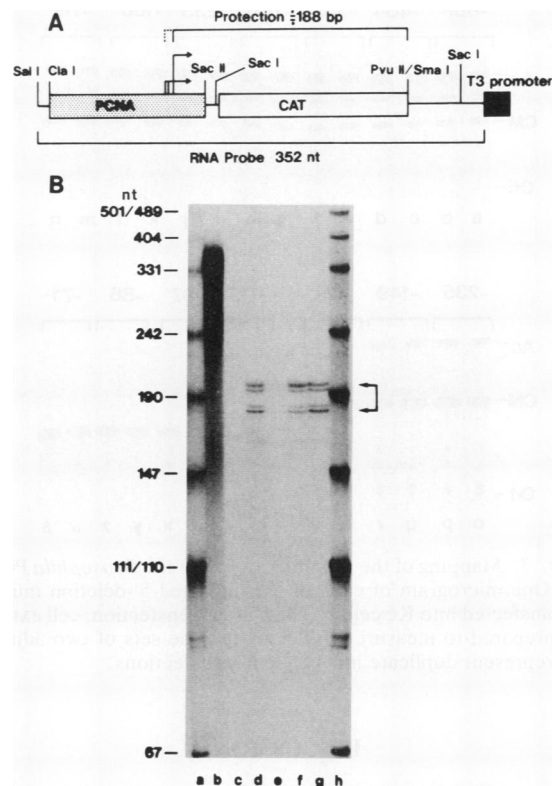
stomodeum invagination shown by short arrows in Fig. 10 (10). In the homozygous *zen* mutant embryos, the germ band extension is incomplete and dorsal tissues are abnormally expanded. The posterior limits of the germ band are shown by long arrows in Fig. 10. Ectopic expression of PCNA was observed in the expanded dorsal region of the *zen* mutant embryo (arrowheads in Fig. 10b).

Similarly, ectopic expression of *lacZ* in the expanded dorsal part was observed in transgenic embryos with the homozygous *zen* mutation (arrowheads in Fig. 10e and h). No apparent difference in *lacZ* expression patterns was observed between embryos carrying either p5'−607DPCN AlacZW8HS or p5'−168DPCNAlacZW8HS. No significant staining with anti-*lacZ* was observed in wild-type and *zen* mutant embryos that did not carry the transgene (Fig. 10f and i). These results indicate that the promoter region of PCNA is responsible for the ectopic expression of *lacZ* in the homozygous *zen* mutant embryo. The fact that ectopic expression was not evident before stage 10 of embryogenesis also suggests that the repression by *zen* is not direct but rather mediated by some unknown process(s).
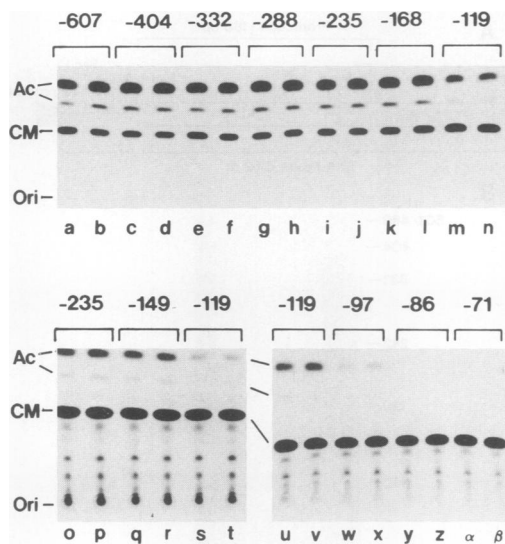
FIG. 7. Mapping of the promoter region of the *Drosophila* PCNA gene. One microgram of each of the indicated 5'-deletion mutants was transfected into Kc cells. At 48 h after transfection, cell extracts were prepared to measure CAT activity. The sets of two adjacent lanes represent duplicate independent transfections.

DISCUSSION

Construction of 5'-deletion derivatives of the PCNA gene in combination with CAT transient expression assays in *Drosophila* Kc cells revealed that promoter function resided within a 192-bp region from positions −168 to +24. The
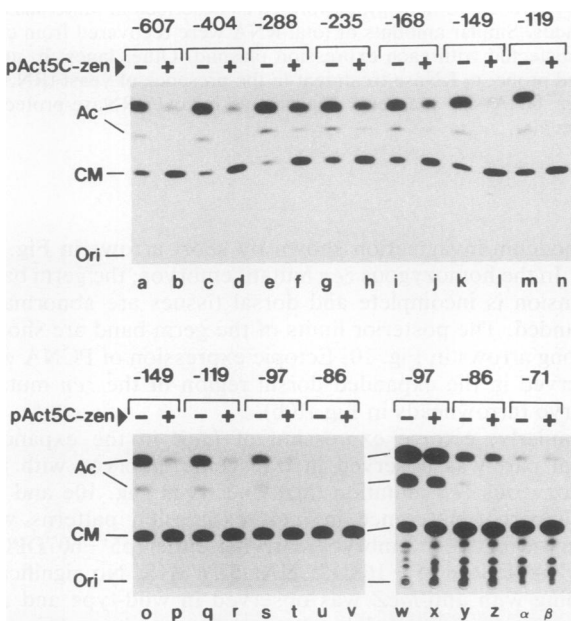
FIG. 8. Mapping of the target region in the PCNA gene for repression by *zen* protein. One microgram of each of the indicated 5'-deletion mutants with (+) or without (−) pAct5C-zen was transfected into Kc cells. At 48 h after transfection, cell extracts were prepared to measure CAT activity. Five times more cell extract was used to measure CAT activity in the experiments shown in lanes w to β.
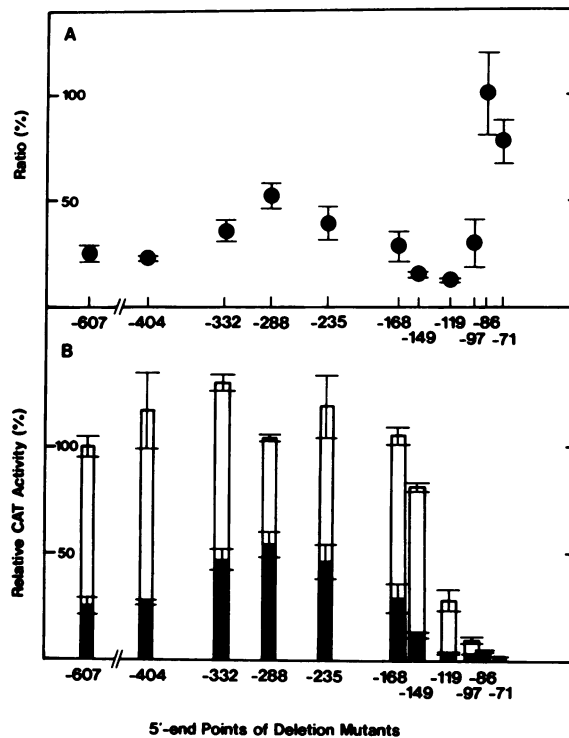

FIG. 9. Quantitative analysis of CAT expression of 5'-deletion mutants with and without *zen*-expressing plasmid. The radioactivities of spots corresponding to acetylated [^{14}C]chloramphenicol on thin-layer chromatographic plates shown in Fig. 7, Fig. 8, and others not shown were quantified. (A) Ratio and standard deviation of CAT expression of each deletion mutant with pAct5C-zen to that without pAct5C-zen. (B) Averages and standard deviations obtained from four independent experiments using 5'-end deletion derivatives with (solid bar) or without (open bar) pAct5C-zen, shown as fractions of CAT expression with p5'−607DPCNACAT alone. The average 100% value was 110,370 cpm.

existence of 10-bp repeats similar to those in the transcription factor Sp1-binding site (19), starting at 42 bp and at 55 bp upstream of the major transcription initiation site, has been reported (Fig. 1). Although the Sp1 homolog has not been detected in *Drosophila* cultured cells (13), these sequences may be involved in the regulation of transcription. We also searched the binding consensus sequences of several reported *Drosophila* transcription factors such as GAGA (5), zeste (3), Adf-1 (23), Cf1 (29), AP1 (39), SRE (7, 36), and actin 5C element (7, 12) and found two sites that perfectly matched the zeste protein-binding consensus 5'-CA/GC TCA/G-3' (4), starting at positions −114 and −51 (Fig. 1). Thus, the *Drosophila* zeste protein may be involved in transcriptional regulation of the PCNA gene.

By cotransfection assay, we examined the *trans*-acting effect of *Drosophila* homeodomain proteins such as those encoded by the *zen*, *eve*, and bicoid genes on the function of the PCNA gene regulatory region. Of these three homeodomain proteins, only the *zen* protein exerted a *trans*-acting effect and repressed expression of the PCNA gene. *zen* is one of the zygotically active genes that control differentiation of the dorsal-ventral pattern during early embryogenesis in *Drosophila* cells (17). During cleavage cycles 10 to 11, *zen* is widely expressed in the dorsal region of the embryo (17). During cellularization and gastrulation, *zen* expression is
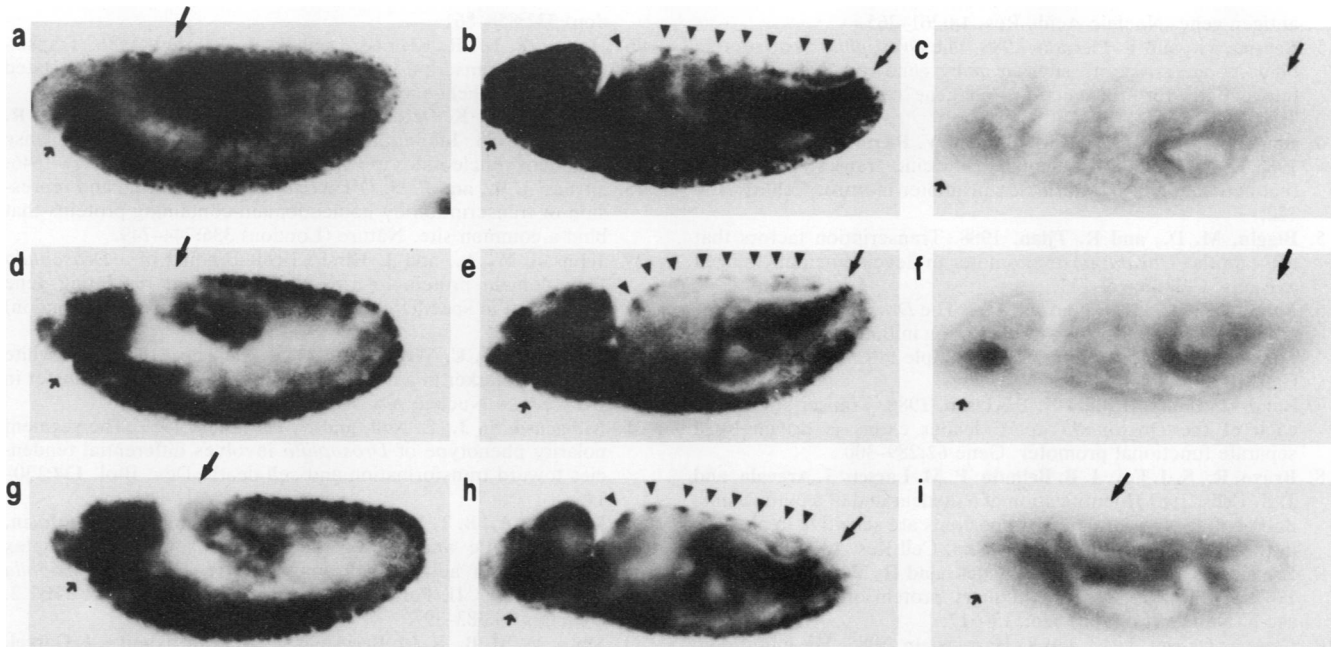
FIG. 10. Expression of *lacZ* directed by the PCNA gene regulatory region in transgenic flies. Expression of *lacZ* from p5'−607DPCNAlacZW8HS in a wild-type embryo (d) or an embryo with a homozygous zen^{w36} mutation (e) and that from p5'−168DPCNA lacZW8HS in a wild-type embryo (g) or an embryo with a homozygous zen^{w36} mutation (h) was visualized by anti-*lacZ* staining. Anti-*lacZ* stains of a nontransgenic wild-type embryo (i) and a nontransgenic embryo with a homozygous zen^{w36} mutation (f) are included as controls. Expression patterns of endogenous PCNA in wild-type and homozygous zen^{w36} mutant embryos detected by immunostaining with anti-PCNA antibody are shown in panels a and b; a zen^{w36} mutant embryo stained with control rabbit IgG is shown in panel c. Ectopic expression in the dorsal regions of embryos homozygous for the *zen* mutation are indicated by arrowheads in panels b, e, and h. The posterior limit of the germ band is shown by long arrows. All of the embryos shown are at early stage 10, which was characterized by the appearance of the stomodeum invagination (short arrows). In all cases, anteriors of the embryos are on the left and internal focal views are shown. Since the homozygous *zen* mutant embryos are slightly twisted, some positive signals in the dorsal part are out of focus. Note that anti-PCNA antibody mainly stained nuclei as dot shapes, while anti-*lacZ* antibody stained cytoplasm. Magnification of each panel is ×185.

restricted to the dorsal-most cells of the middle-body region of the embryo, where, according to the embryo fate map (49), the presumptive optic lobe and amnioserosa are located. This region also coincides quite closely with the so-called mitotic domain A (21). It was reported that cells in mitotic domain A do not divide after the cleavage cycle 14 (21). Thus, in nondividing cells in this region, the expression of *zen* protein may activate the mechanism that can maintain the DNA replication enzymes at a very low level throughout embryogenesis by repressing their expression. In fact, zygotic expression of PCNA-*lacZ* was very low in the dorsal-most cells of the gastrulating embryos from transgenic flies, and ectopic expression of PCNA-*lacZ* in the dorsal region was observed in embryos homozygous for the zen^{w36} mutation (Fig. 10). Target genes of *zen* have been assumed to be those that promote differentiation of dorsal cells to the amnioserosa and optic lobe. The results described in this report also indicate that the PCNA gene is another target of *zen*. The mechanism that activates genes for differentiation and simultaneously inactivates genes for DNA replication to block cell proliferation might be an effective way for cells to promote differentiation.

As shown in Fig. 9A, the removal of homeodomain protein-binding sites modulated only marginally the extent of repression by *zen* protein, and removal of the region between −119 and −86, which is in the distal portion of the promoter, completely abolished the effect of the *zen* protein. Since we have not observed any binding of *zen* protein to this promoter region (52), the repressive effect of *zen* protein

on this region would be indirect. *zen* may regulate the level of some unknown transcription factor(s) that binds to this region (−119 to −86) of the PCNA promoter. The results of in vivo analysis with transgenic flies support this conclusion, since the PCNA promoter without upstream *zen*-binding sites is fully responsible for the ectopic expression in the dorsal region of *zen* mutants.

REFERENCES

1. **Almendral, J. M., D. Huebsh, P. A. Blundell, H. MacDonald-Bravo, and R. Bravo.** 1987. Cloning and sequence of the human nuclear protein cyclin: homology with DNA-binding proteins. Proc. Natl. Acad. Sci. USA **84:**1575–1579.
2. **Bauer, G. A., and P. M. J. Burgers.** 1990. Molecular cloning, structure and expression of the yeast proliferating cell nuclear

antigen gene. Nucleic Acids Res. **18:**261–265.

3. **Benson, M., and V. Pirrotta.** 1988. The *Drosophila zeste* protein binds cooperatively to sites in many gene regulatory regions: implications for transvection and gene regulation. EMBO J. **7:**3907–3915.

4. **Biggin, M. D., S. Bickel, M. Benson, V. Pirrotta, and R. Tjian.** 1988. *Zeste* encodes a sequence-specific transcription factor that activates the *Ultrabitholax* promoter in vitro. Cell **53:**713–722.

5. **Biggin, M. D., and R. Tjian.** 1988. Transcription factors that activate the *Ultrabithorax* promoter in developmentally staged extracts. Cell **53:**699–711.

6. **Bond, B. J., and N. Davidson.** 1986. The *Drosophila melanogaster* actin 5C gene uses two transcription initiation sites and three polyadenylation sites to express multiple mRNA species. Mol. Cell. Biol. **6:**2080–2088.

7. **Bond-Matthews, B., and N. Davidson.** 1988. Transcription from each of the *Drosophila act5C* leader exons is driven by a separate functional promoter. Gene **62:**289–300.

8. **Bravo, R., S. J. Fey, J. B. Bellatin, P. M. Larsen, J. Arevalo, and J. E. Celis.** 1981. Identification of a nuclear and of a cytoplasmic polypeptide whose relative proportions are sensitive to changes in the rate of cell proliferation. Exp. Cell Res. **136:**311–319.

9. **Bravo, R., R. Frank, P. A. Blundell, and H. MacDonald-Bravo.** 1987. Cyclin/PCNA is the auxiliary protein of DNA polymerase-δ. Nature (London) **326:**515–517.

10. **Campos-Ortega, J. A., and V. Hartenstein.** 1985. The embryonic development of *Drosophila melanogaster.* Springer-Verlag, Berlin.

11. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18:**5294–5299.

12. **Chung, Y.-T., and E. B. Keller.** 1990. Regulatory elements mediating transcription from *Drosophila melanogaster* actin 5C proximal promoter. Mol. Cell. Biol. **10:**206–216.

13. **Courey, A. J., and R. Tjian.** 1988. Analysis of Sp1 *in vivo* reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. Cell **55:**887–898.

14. **Cross, D. P., and J. H. Sang.** 1978. Cell culture of individual *Drosophila* embryos. I. Development of wild-type cultures. J. Embryol. Exp. Morphol. **45:**161–172.

15. **Desplan, C., J. Theis, and P. H. O'Farrell.** 1988. The sequence specificity of homeodomain-DNA interaction. Cell **54:**1081–1090.

16. **Di Nocera, P. P., and I. B. Dawid.** 1983. Transient expression of genes introduced into cultured cells of *Drosophila.* Proc. Natl. Acad. Sci. USA **80:**7095–7098.

17. **Doyle, H. J., R. Kraut, and M. Levine.** 1989. Spatial regulation of *zerknüllt*: a dorsal-ventral patterning gene in *Drosophila.* Genes Dev. **3:**1518–1533.

18. **Driever, W., and C. Nüsslein-Volhard.** 1989. The bicoid protein is a positive regulator of *hunchback* transcription in the early *Drosophila* embryo. Nature (London) **337:**138–143.

19. **Dynan, W. S., S. Sazer, R. Tjian, and R. T. Schimke.** 1986. Transcription factor Sp1 recognizes a DNA sequence in the mouse dihydrofolate reductase promoter. Nature (London) **319:**246–248.

20. **Echalier, G., and A. Ohanessian.** 1970. In vivo culture of *Drosophila melanogaster* embryonic cells. In Vitro **6:**162–172.

21. **Foe, V.** 1989. Mitotic domains reveal early commitment of cells in *Drosophila* embryos. Development **107:**1–22.

22. **Han, K., M. S. Levine, and J. L. Manley.** 1989. Synergistic activation and repression of transcription by Drosophila homeobox proteins. Cell **56:**573–583.

23. **Heberlein, U., B. England, and R. Tjian.** 1985. Characterization of *Drosophila* transcription factors that activate the tandem promoters of the alcohol dehydrogenase gene. Cell **41:**965–977.

24. **Hiromi, Y., A. Kuroiwa, and W. J. Gehring.** 1985. Control elements of *Drosophila* segmentation gene *fushi tarazu.* Cell **43:**603–613.

25. **Hoey, T., and M. Levine.** 1988. Divergent homeobox proteins recognize similar DNA sequences in *Drosophila.* Nature (Lon-

don) **332:**858–861.

26. **Hultmark, D., R. Klemenz, and W. J. Gehring.** 1986. Translational and transcriptional control elements in the untranslated leader of the heat-shock gene hsp 22. Cell **44:**429–438.

27. **Jaskulski, D., K. DeRiel, W. E. Mercer, B. Calabretta, and R. Baserga.** 1988. Inhibition of cellular proliferation by antisense oligodeoxynucleotides to PCNA/cyclin. Science **240:**1544–1546.

28. **Jaynes, J. B., and P. H. O'Farrell.** 1988. Activation and repression of transcription by homeodomain-containing proteins that bind a common site. Nature (London) **336:**744–749.

29. **Johnson, W. A., and J. Hirsh.** 1990. Binding of a *Drosophila* POU-domain protein to a sequence element regulating gene expression in specific dopaminergic neurons. Nature (London) **343:**467–470.

30. **Klements, R., U. Weber, and W. J. Gehring.** 1987. The white gene as a marker in a new P-element vector for gene transfer in *Drosophila.* Nucleic Acids Res. **15:**3947–3959.

31. **Klingensmith, J., E. Noll, and N. Perrimon.** 1989. The segment polarity phenotype of *Drosophila* involves differential tendencies toward transformation and cell death. Dev. Biol. **134:**130–145.

32. **Lewis, R. A., B. T. Wakimoto, R. E. Denell, and T. C. Kaufman.** 1980. Genetic analysis of the antennapedia gene complex (ANT-C) and adjacent chromosomal regions of *Drosophila melanogaster.* II. Polytene chromosome segments 84A-84B1,2. Genetics **95:**383–397.

33. **Mathews, M. B., R. M. Bernstein, B. R. Franza, and J. I. Garrel.** 1984. Identity of the proliferating cell nuclear antigen and cyclin. Nature (London) **309:**374–376.

34. **Matsumoto, K., T. Moriuchi, T. Koji, and P. K. Nakane.** 1987. Molecular cloning of cDNA coding for rat proliferating cell nuclear antigen (PCNA)/cyclin. EMBO J. **6:**637–642.

35. **Miyachi, K., M. J. Fritzler, and E. M. Tan.** 1978. Autoantibody to a nuclear antigen in proliferating cells. J. Immunol. **121:**2228–2234.

36. **Mohun, T., N. Garnett, and R. Treisman.** 1987. *Xenopus* cytoskeletal actin and human c-fos gene promoters share a conserved protein-binding site. EMBO J. **6:**667–673.

37. **Müller, M., M. Affolter, W. Leupin, G. Otting, K. Wuthrich, and W. J. Gehring.** 1988. Isolation and sequence-specific DNA binding of the *Antennapedia* homeodomain. EMBO J. **7:**4299–4304.

38. **Ng, L., G. Prelich, C. W. Anderson, B. Stillman, and P. A. Fisher.** 1990. *Drosophila* proliferating cell nuclear antigen; structural and functional homology with its mammalian counterpart. J. Biol. Chem. **265:**11948–11954.

39. **Perkins, K. K., G. M. Dailey, and R. Tjian.** 1988. Novel Jun- and Fos-related proteins in *Drosophila* are functionally homologous to enhancer factor AP-1. EMBO J. **7:**4265–4273.

40. **Prelich, G., M. Kostura, D. R. Marshak, M. B. Mathews, and B. Stillman.** 1987. The cell-cycle regulated proliferating cell nuclear antigen is required for SV40 DNA replication *in vitro.* Nature (London) **326:**471–475.

41. **Prelich, G., and B. Stillman.** 1988. Coordinated leading and lagging strand synthesis during SV40 DNA replication *in vitro* requires PCNA. Cell **53:**117–126.

42. **Prelich, G., C. K. Tan, M. Kostura, M. B. Mathews, A. G. So, K. M. Downey, and B. Stillman.** 1987. Functional identity of proliferating cell nuclear antigen and a DNA polymerase δ auxiliary protein. Nature (London) **326:**517–520.

43. **Robertson, H. M., C. R. Preston, R. W. Phillis, D. M. Johnson-Schlitz, W. K. Benz, and W. R. Engels.** 1988. A stable genomic source of P-element transposase in *Drosophila melanogaster.* Genetics **118:**461–470.

44. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed., p 1.38–1.43, 7.14, 7.71–7.79. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

45. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.

46. **Spradling, A. C.** 1986. P-element-mediated transformation, p. 175–197. *In* D. B. Roberts (ed.), Drosophila: a practical ap-

proach. IRL Press, Oxford.

47. Suzuka, I., H. Daidoji, M. Matsuoka, K.-I. Kadowaki, Y. Takasaki, P. K. Nakane, and T. Moriuchi. 1989. Gene for proliferating-cell nuclear antigen (DNA polymerase δ auxiliary protein) is present in both mammalian and higher plant genomes. Proc. Natl. Acad. Sci. USA 86:3189–3193.

48. Takasaki, J., D. Fischwild, and E. M. Tan. 1981. Characterization of proliferating cell nuclear antigen recognized by autoantibodies in lupus sera. J. Exp. Med. 159:981–992.

49. Wakimoto, B. T., F. R. Turner, and T. C. Kaufman. 1984. Defects in embryogenesis in mutants associated with the Antennapedia gene complex of Drosophila melenogaster. Dev. Biol. 102:147–172.

50. Yamaguchi, M., Y. Hayashi, F. Hirose, S. Matsuoka, T. Moriu-

chi, T. Shiroishi, K. Moriwaki, and A. Matsukage. 1991. Molecular cloning and structural analysis of mouse gene and pseudogenes for proliferating cell nuclear antigen. Nucleic Acids Res. 19:2403–2410.

51. Yamaguchi, M., Y. Hayashi, and A. Matsukage. 1988. Mouse DNA polymerase β gene promoter: fine mapping and involvement of Sp1-like mouse transcription factor in its function. Nucleic Acids Res. 16:8773–8787.

52. Yamaguchi, M., Y. Nishida, T. Moriuchi, F. Hirose, C.-C. Hui, Y. Suzuki, and A. Matsukage. 1990. Drosophila proliferating cell nuclear antigen/cyclin gene: structure, expression during development, and specific binding of homeodomain proteins to its 5'-flanking region. Mol. Cell. Biol. 10:872–879.