The Drosophila proliferating cell nuclear antigen gene promoter contains multiple transcriptional regulatory elements, including upstream regulatory element (URE), DNA replication-related element, E2F recognition sites, and three common regulatory factor for DNA replication and DNA replication-related element-binding factor genes recognition sites. In nuclear extracts of Drosophila embryos, we detected a protein factor, the URE-binding factor (UREF), that recognizes the nucleotide sequence 5′-AAACCAGTTGGCA located within URE. Analyses in Drosophila Kc cells and transgenic flies revealed that the UREF-binding site plays an important role in promoter activity both in cultured cells and in living flies. A yeast one-hybrid screen using URE as a bait allowed isolation of a cDNA encoding a transcription factor, Grainyhead/nuclear transcription factor-1 (GRH/NTF-1). The nucleotide sequence required for binding to GRH was indistinguishable from that for UREF detected in embryo nuclear extracts. Furthermore, a specific antibody to GRH reacted with UREF in embryo nuclear extracts. From these results we conclude that GRH is identical to UREF. Although GRH has been thought to be involved in regulation of differentiation-related genes, this study demonstrates, for the first time, involvement of a GRH-binding site in regulation of the DNA replication-related proliferating cell nuclear antigen gene.

The proliferating cell nuclear antigen (PCNA)1 is required for replication of simian virus 40 (SV40) (1) as well as cellular DNA (2, 3). It has been proposed to function as a sliding clamp at DNA replication forks (4) and is also involved in DNA repair (5, 6) and cell cycle regulation (7–9) by interacting with various enzymes and regulatory proteins (10, 11). A possible role of PCNA in marking of DNA for chromatin assembly has also been proposed (12, 13).

In previous studies of the Drosophila genes for PCNA and DNA polymerase α, we found a common 8-base pair sequence named the DNA replication-related element (DRE) (14–16), which appeared to be an important regulatory element not only for these two DNA replication-related genes but also for various other cell cycle- (17) and cell proliferation-related genes (18, 19). We also identified a specific DRE-binding factor (DREF) in Drosophila melanogaster (14). The cDNAs and genes for D. melanogaster (20) and Drosophila virilis (21) DREF proteins have been cloned and characterized. Various in vivo experiments have revealed that DREF is essential for the function of the PCNA promoter both in embryos and in larvae (15, 22).

We have also identified two E2F recognition sites (nucleotide positions –56 to –36 with respect to the cap site) in the region downstream of the PCNA gene DRE (–100 to –93) (23). Multiple E2F sites have been also identified in the promoters of the Drosophila DNA polymerase α 180-kDa subunit (24) and the 73-kDa subunit (16). In mammals, E2F and its heterodimeric partner DP associate to E2F sites for activation of the target genes (25). cDNAs have been cloned for the Drosophila counterparts de2F, de2F2, and ddP (26–28). Transcription of DNA polymerase α and PCNA genes is completely lost in de2F or ddP mutant embryos after division cycle 16, indicating that de2F and ddP are essential for transcription of these DNA replication-related genes (29, 30). The function of de2F2 has yet to be determined. Analyses with transgenic flies demonstrated that two E2F sites are essential for PCNA promoter activity throughout development (23). However, E2F sites alone proved to be insufficient for PCNA promoter activity during embryonic and larval stages, because deletion of the upstream region containing DRE completely abolished the promoter activity during these stages (23).

In addition to DRE and E2F sites, the PCNA promoter contains three common regulatory factor for DNA replication and DREF genes (CFDD) recognition sites, site 1 (−84 to −77), site 2 (−100 to −93), and site 3 (−134 to −127) (31). Among these three, at least site 1 could be demonstrated to play an important role in promoter activity in both cultured cells and living flies (31). In addition to the PCNA gene, multiple CFDD sites were found in promoters of the DNA polymerase α and DREF genes (31).

Another important regulatory element for the PCNA promoter is the upstream regulatory element (URE) located in the region from nucleotide positions –168 to –119 (22). URE, in addition to the E2F sites, CFDD sites, and DRE, appears to be essential for activation of the PCNA promoter in larvae (22). A
protein factor(s), which specifically binds to URE, has hitherto not been identified. In the present study, we detected one such binding factor, the URE-binding factor (UREF) that recognizes the sequence 5′-AAACCAGTGGCA. A yeast one-hybrid screening using URE as the bait allowed isolation of a cDNA encoding a transcription factor, Grainyhead (GRH/NTF-1) (32, 33). Several lines of evidence indicate that GRH is a likely candidate for UREF with possible roles in regulation of the PCNA promoter in vivo.

EXPERIMENTAL PROCEDURES

Oligonucleotides—The sequences of double-stranded oligonucleotides containing DRE (DRE-P) or E2F sites (E2F-P) in the PCNA gene promoter were defined as follows.

| Sequence | Description |
|----------|-------------|
| 3UREwt | containing DRE site 1 (DPCNA) |
| 3UREmut | containing DRE site 1 (DPCNA) |

For target sequences in the cotransfection experiments, the following double-stranded oligonucleotides were used.

| Sequence | Description |
|----------|-------------|
| 3UREwt | containing DRE site 1 (DPCNA) |
| 3UREmut | containing DRE site 1 (DPCNA) |

Plasmid Construction—The plasmid p5′-149DPCNACAT contains the PCNA gene fragment spanning from −149 to +24 placed upstream of the chloramphenicol acetyltransferase (CAT) gene in the plasmid pHSKCAT (34).

A fragment from −149 to +24 having four base-substituted mutations was generated by the polymerase chain reaction method using p5′-149DPCNACAT as a template with primers mutβ and CAT-1 (31). The polymerase chain reaction product was blunt-ended with a DNA blunting kit (Takara) and digested with SacI. The p5′-149DPCNACAT was digested with SacI (−149), blunt-ended with the Mung bean nuclease, digested with SacI, and then ligated with the polymerase chain reaction product to create the plasmid p5′-149mutβDPCNACAT. p5′-149mutβDPCNACAT and p5′-149mutDPCNACAT were created in a similar way except that primers mut and mutβ were used for the polymerase chain reaction, respectively. p3UREwt-TATACAT and p3UREmutβ-TATACAT were respectively created by ligating the double-stranded oligonucleotides 3UREwt and 3UREmutβ into the SacI site of pTATA-SolICAT. pTATA-SolICAT contains Rpnl, Apal, Xhol, SalI, and SpeI sites in front of the metallothionein gene basal promoter containing a TATA box and the cap site (14).

The plasmid grhNpNB40 (35) containing the N form of GRH cDNA was cut with XhoI and blunt-ended, and the NotI linker was added. Then the DNA fragment containing the grhN cDNA was cut out with NotI and inserted into the NotI site of pGEM-Actex3 (36) to create pAct-GRH(N). pAct-GRH(O) was created in the same way except that the plasmid grhO/pNB40 (37) containing the O form of GRH cDNA was used.

For target sequences in the cotransfection experiments, the following double-stranded oligonucleotides were used.

| Sequence | Description |
|----------|-------------|
| 3UREwt | containing DRE site 1 (DPCNA) |
| 3UREmut | containing DRE site 1 (DPCNA) |

Sequences 13 and 14

Expression of GST Fusion Proteins—Expression of GST-GRH fusions in E. coli XL-1 Blue was carried out as described elsewhere (40). Lysates of cells were prepared by sonication in buffer D containing 0.6 M KCl, 1 mM phenylmethylsulfonyl fluoride, 1 μg each of pepstatin, leupeptin, and aprotinin. Lysates were cleared by centrifugation at 12,000 × g for 20 min at 4 °C and applied to glutathione-Sepharose (Amersham Pharma Biotech) to purify the GST-GRH fusion protein as described elsewhere (40). The GST protein was expressed and purified in the same way.

Antibodies—The purified GST-GRH fusion protein and the purified GST protein were used to elicit polyclonal antibody production in mice. Polyclonal antibodies reacting with GST-GRH or GST were purified from antisera using E2-SEP (Amersham Pharma Biotech).

One-hybrid Screening—The MATCHAKER one-hybrid system protocol was used to prepare the target reporter constructs, to integrate these constructs into Saccharomyces cerevisiae strain YM4271 (his “ura” leu”), and to screen the activation domain fusion library (AAct-cDNA library) from Drosophila instar larvae (kindly supplied by Dr. Elledge). Five tandem copies of the double-stranded oligonucleotide UREL (5′UREL) were placed upstream of the marker genes of both the pHIS1-1 and pLaCZi plasmids (CLONTECH). The two target reporter constructs were transformed into S. cerevisiae strain.
YM4271 in a consecutive manner to produce a dual reporter strain. This was transformed with the pACT-dDNA library and his ['ura' leu] transformants grown in synthetic dropout medium containing 40 mg/ml 3-amino-nitroazole were selected. Each colony was streaked on synthetic dropout agar medium without histidine but containing 40 mg/ml 3-amino-nitroazole and incubated for 3 days at 30 °C. A dry NEF-975X filter (NEN Life Science Products) was placed over the surface of each agar plate containing transformants. The filters were lifted off the agar plate and dipped in liquid nitrogen. The frozen colonies were then thawed at room temperature and the filters were overlaid onto Whatman 5 filters that had been soaked in Z buffer (60 mM NaHPO4, 7.0H2O, 40 mM Na2HPO4, 6.8O, 0.5 mM dithiothreitol, 0.05 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 2 μg/ml pepstatin, 0.75 μg/ml aprotinin). The homogenates were filtered through two layers of nylon cloth, and the filtrate was centrifuged for 10 min at 400 × g to remove debris. Nuclei were pelleted from the supernatant by centrifugation for 10 min at 4500 × g. The pellet was resuspended in 10 ml of 1 mM sucrose/solution I. Then 5 ml each were layered on a 26 ml gradient of 1.0–1.7 M sucrose/solution I, and nuclei were centrifuged for 15 min at 17,000 × g in a swinging bucket rotor. The pellet was resuspended in 3 volumes of solution II (10 mM HEPES (pH 7.9), 0.4 mM NaCl, 3 mM MgCl2, 0.5 mM DTT, 5% glycerol, with protease inhibitors as above) and incubated for protein extraction. Specific DNA-protein complexes were used for band mobility shift analysis. With this oligonucleotide 4 °C for 30 min before centrifugation at 100,000 × g for 5 min. The supernatant was dialyzed in solution III (20 mM HEPES (pH 7.9), 50 mM NaCl, 0.5 mM DTG, 0.5 mM phenylmethylsulfonyl fluoride, 20% glycerol), clarified by centrifugation, and stored at –80 °C in small aliquots. The protein concentration of the extracts was 2.1 mg/ml.

### Band Mobility Shift Assay

Band mobility shift analysis was performed as described earlier (14) with minor modifications. Two μl of 32P-labeled probes (10,000 cpm) were mixed with 10.25 μl of binding buffer containing 25 mM HEPES (pH 7.6), 100 mM KCl, 1 mM EDTA, 1 mM MgCl2, 0.5 μM DTT, 5% glycerol, and 0.5 μl of 1 mg/ml calf thymus DNA were added, and then the probe mixture was incubated on ice for 5 min. When necessary, unlabelled oligonucleotides were added as competitors at this step. Purified GST-GRH fusion protein was diluted with buffer containing 25 mM HEPES (pH 7.6), 600 mM KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol. Two μl (0.1 μg) of each fusion protein was added to the probe mixture and then incubated for 15 min at room temperature. 4.2 μg of Drosophila embryo nuclear extracts were incubated in the same reaction mixture without calf thymus DNA. In experiments with antibodies, the antibody was either preincubated with embryo nuclear extracts for 2 h before reaction with DNA probes or incubated after the binding reaction between nuclear extracts and DNA probes for 2 h on ice. DNA-protein complexes were electrophoretically resolved on 4% polyacrylamide gels in 100 mM Tris borate (pH 8.3), 2 mM EDTA containing 2.5% glycerol at 25 °C. The gels were dried and then autoradiographed.

### DNA Transfection into Cells, CAT Assays, and Luciferase Assays—

Drosophila Kc cells (41) were grown in M3(BF) medium supplemented with 2% fetal calf serum (42) and plated at about 2 × 105 Kc cells (41) were grown in M3(BF) medium supplemented with 2% fetal calf serum (42) and plated at about 2 × 105 Kc cells (41) were grown in M3(BF) medium supplemented Drosophila Drosophila were grown in M3(BF) medium supplemented with 2% fetal calf serum (42) and plated at about 2 × 105 Kc cells (41) were grown in M3(BF) medium supplemented Drosophila Drosophila dried and then autoradiographed.

### Figure 1. Organization of PCNA promoter elements.

The vertical line with a horizontal arrow indicates the transcription initiation site. The E2F site, DRE, URE, and CFDD sites are shown. Locations of each site relative to the cap site are indicated by numbers with vertical lines.

The luciferase assay was carried out with a PicaGene assay kit (Toyo Ink) as described previously (45). All assays were performed within the range of linear relation of activity to incubation time and protein amount. CAT activity was normalized to the luciferase activity or protein amount determined by Bio-Rad protein assay. The obtained values were essentially comparable with those normalized to protein amounts.

### Establishment of Transgenic Flies and Analysis of PCNA-lacZ Expression Patterns—

Fly stocks were maintained at 25 °C on standard food. Canton S flies were used as the wild type strain. P-element mediated germ line transformation was carried out as described earlier (46) and G0 transgenic flies were transformed on the basis of white eye color rescue (47). Multiple independent lines were obtained for each of the various fusion genes. Established transgenic fly strains and their chromosome linkages are listed in Table 1. Quantitative measurement of β-galactosidase activity in extracts was carried out as described previously (48).

### RESULTS

#### Detection of URE-binding Factor—

The Drosophila PCNA gene promoter is regulated by multiple transcriptional regulatory elements URE (Fig. 1). URE stimulates the PCNA gene promoter activity in cultured Drosophila Kc cells and in embryos (22, 34). Furthermore, URE is essential for the PCNA gene promoter activity in larvae (22). To detect a factor(s) binding to URE, an oligonucleotide containing the region from –149 to –118 (UREL) (Fig. 1) was chemically synthesized and used for band mobility shift analysis. With this oligonucleotide and embryo nuclear extracts, specific DNA-protein complexes were detected, which were diminished by adding an excess amount of unlabelled UREL oligonucleotide as a competitor but not by adding oligonucleotides DRE-P and DRE-PM (Fig. 3).

The results suggest that a factor different from DREF can bind to URE. UREF

#### Nucleotide Sequences Required for Binding to UREF—

To determine the nucleotide sequence required for binding to UREF, various base substitution mutations were introduced in the region between –149 and –118 (Fig. 2), and the resultant mutant oligonucleotides were used as competitors in the band mobility shift analysis. The mutant oligonucleotide mutants only weakly competed for the binding (Fig. 4, lanes f–h). The mutant oligonucleotide mutants and mutations did not compete at all (Fig. 4, lanes i–n), whereas the other mutant oligonucleotides competed for the binding as effectively as the wild type UREL oligonucleotide (Fig. 4, lanes b–e and m–o). These results indicate that the sequence 5′-AAACCAAGTTGGCA plays an important role in UREF binding (Fig. 2).

### Effects of Mutations in the UREF-binding Site on PCNA Promoter Activity in Kc Cells—

To examine effects of mutations in the UREF-binding site on PCNA promoter activity, base substitution mutations were introduced in and around the site,
and the mutated promoter was placed upstream of the CAT gene in a CAT vector. Plasmids carrying these constructs were then transfected into Kc cells, and CAT expression levels were determined. As shown in Fig. 5, mutations in the URE-binding site on p5′-149mutDPCNA CAT and p5′-149mutβDPCNA CAT reduced the CAT expression to 46 and 61%, respectively. However, the mutation outside of the URE-binding site (p5′-149mutDPCNA CAT) exerted no effect on the CAT expression. These results indicate that the URE-binding site plays an important role in PCNA promoter activity in Kc cells.

Role of the URE-Binding Site in the Function of the PCNA Promoter in Living Flies—Although the results of CAT transient expression assay in Kc cells clearly demonstrated an important role of URE-binding site for PCNA promoter activity, these observations needed to be further confirmed in living flies. For this purpose, transgenic Drosophila provide an appropriate system to characterize transcriptional regulatory elements in vivo.

Previously, we established transgenic flies carrying PCNA (−149 to +137 or −119 to +137) and lacZ fusion genes (22). To examine the role of the URE-binding site in the PCNA promoter activity during Drosophila development, we generated PCNA-lacZ fusion genes carrying base substitution mutations in and around the URE-binding site. These fusion genes were then introduced into flies by germ line transformation. Established transgenic lines and their chromosomal linkages are listed in Table I. Activities of the modified promoters were then monitored by quantitative β-galactosidase assay at various developmental stages.

In flies carrying the PCNA gene promoter region up to position −149, a 4-base substitution in the URE-binding site (mutβ, p5′-149mutβDPCNA lacZWSHS) reduced the lacZ expression in embryo and larvae (Fig. 6). Similarly, in flies carrying the PCNA promoter region up to position −119 (p5′-119DPCNA lacZWSHS), the lacZ expression was reduced (Fig. 6). Because the extent of the reduction was most prominent in larvae, β-galactosidase activity was demonstrated in dissected larval tissues (Fig. 7). In transgenic third instar larvae carrying a wild type construct, high lacZ-staining signal was observed in the salivary glands (A) and probable neuroblasts in the central nervous system (E). The larvae having a mutation in the URE-binding site (mutβ) had a reduced staining signal in the salivary glands (B) and almost no staining signals were observed in the central nervous system (F). A deletion in position −119 almost completely abolished the staining signals in both tissues (D and H). In contrast, no reduction of staining was observed with lines carrying a mutation outside the URE-binding site (mutα) (C and O). Thus, an important role of the URE-binding site for PCNA promoter activity was confirmed in living flies.

Molecular Cloning of cDNA Encoding for the Protein Responsible for the URE Binding Activity—We used a yeast one-hybrid screen to isolate a cDNA encoding the protein responsible for the URE binding activity. We screened a Drosophila third instar larva cDNA library using five tandem copies of the URE sequence (UREL) as the target binding sequence. After screening 1.5 × 10^7 independent clones, seven independent positive clones were identified, all of which were partially sequenced. All seven clones were found to have 99% identity with a cDNA encoding GRH/NTF-1 (32) (GenBank™ Accession number X15657). Four GRH isoforms (N, N, O, and O) corresponding to the O form of GRH resides between amino acids 873 and 1132, the protein encoded by the cloned cDNA consisted of the DNA-binding domain. This clone, designated as pACT-GRH438–1333, was then transformed into yeast containing the 5UREL-reporter, the 3URE-reporter, or four copies of the CFDD-1 (4CFDD) reporter, or four copies of the E2F-P (4E2F) reporter. As shown in

FIG. 2. Nucleotide sequences in and around the URE-binding site in the Drosophila PCNA gene. Nucleotide sequences of mutant oligonucleotides are shown. Nucleotides substituted for the wild type sequence are shaded. The sequences for the URE-binding site and CFDD-binding site 3′ (31) are indicated by brackets.

Fig. 3. Detection of URE binding activity in embryo nuclear extracts by band mobility shift assay. Radiolabeled double-stranded URE oligonucleotides were incubated with embryo nuclear extract (4.2 μg of protein) in the presence or absence (0) of the indicated amounts of competitor oligonucleotides (indicated at the top of each lane).

Fig. 4. Effects of mutations in the URE on complex formation between URE oligonucleotides and embryo nuclear extracts. Radiolabeled double-stranded URE oligonucleotides were incubated with embryo nuclear extract (4.2 μg of protein) in the presence or absence (0) of the indicated amounts of mutant oligonucleotides (mutα to mutθ) are shown in Fig. 2.
Fig. 8, only with the 5UREL and 3URE reporters did the transformants grow in the presence of 40 mM 3-aminotriazole. The results indicate that the GAL4 (activation domain)-GRH438–1333 fusion protein specifically interacts with the URE sequence in yeast cells.

Nucleotide Sequence Required for Binding to GST-GRH Fusion Protein—The GST-GRH873–1333 fusion protein containing the DNA-binding domain and the following C-terminal region of GRH was purified from bacteria expressing the fusion protein. The SDS-polyacrylamide gel electrophoresis profile of the purified fusion protein is shown in Fig. 9. This highly purified preparation was used for the band mobility shift analyses using UREL oligonucleotide as a probe. As shown in Fig. 10, GST-GRH873–1333 fusion protein specifically bound to UREL, but not to oligonucleotides containing DRE (DRE-P) or CFDD-binding site 1 (CFDD-1). The same set of mutant oligonucleotides (Fig. 2) used for the analyses of binding specificity of UREF in the embryo nuclear extracts was applied to the band mobility shift analyses using GST-GRH873–1333. As shown in Fig. 11, the mutant oligonucleotides mutα only weakly competed for the binding (lanes e–g), and mutant oligonucleotides mutβ and mutγ did not compete at all (Fig. 11, lanes h–n). In contrast, the other mutant oligonucleotides mutδ and mutε competed for the binding as effectively as the wild type UREL oligonucleotide (Fig. 11, lanes a–d and o–t). Therefore the nucleotide sequence required for binding to GST-GRH873–1333 was determined to be 5′-AAACCAGTTGGCA, indistinguishable from that for UREF.

GRH Is Identical to UREF—The similarity in DNA-binding specificities between GRH and UREF suggested identity of the two. We prepared an anti-GRH antibody and added it to the band mobility shift assay using Drosophila embryo nuclear extracts and the UREL oligonucleotide probe. As shown in Fig. 12, preincubation of the anti-GRH IgG with the extracts inhibited complex formation between UREF and the UREL probe (lanes e–g), whereas addition of the anti-GRH IgG after the binding reaction supershifted the UREF-UREL complex (lanes

### Table I

| P-element plasmid            | Strain | Chromosome linkage |
|-------------------------------|--------|-------------------|
| p5′-149DPCNAlacZW8HS          | 6      | III               |
|                               | 20     | II                |
|                               | 25     | III               |
|                               | 30     | III               |
|                               | 38     | III               |
|                               | 87     | III               |
|                               | 94     | III               |
| p5′-149mutβDPCNAlacZW8HS      | 8      | II                |
|                               | 36     | III               |
|                               | 45     | III               |
|                               | 52     | II                |
| p5′-149mutδDPCNAlacZW8HS      | 7      | III               |
|                               | 14     | II                |
|                               | 15     | III               |
|                               | 47     | II                |
| p5′-119DPCNAlacZW8HS          | 29     | III               |
|                               | 30     | III               |
|                               | 40     | III               |
|                               | 67     | III               |
| p5′-119mutβDPCNAlacZW8HS      | 21     | II                |
|                               | 35     | III               |
|                               | 50     | II                |
|                               | 77     | III               |

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

Fig. 6. Effects of mutations in and around the UREF-binding site on PCNA promoter activity in transgenic flies. Male transgenic flies (indicated in each panel) were crossed with female wild type flies, and extracts were prepared from Drosophila bodies at various stages of development. The β-galactosidase activities in the extracts are expressed as absorbance units/h/mg of protein. Closed bars indicate the average values for independent transgenic strains carrying the indicated fusion gene. Numbers (n) of independent strains carrying the same fusion gene are given in each panel.

5′-AAACCAGTTGGCA, indistinguishable from that for UREF.
In both reactions, control IgG (Fig. 12, lanes a–d and h–k) or anti-GST IgG (data not shown) exerted no effects on the complex formation. These results indicated GRH to be identical to UREF.

Effects of GRH on PCNA Promoter Activity—To determine whether the PCNA promoter can be activated by GRH, cotransfection assays using Kc cells were carried out. Expression of the N form of GRH (GRH(N)) or the O form (GRH(O)) slightly stimulated PCNA promoter-directed CAT expression (Fig. 13A). When the PCNA promoter carrying a mutation in the GRH(UREF)-binding site was used as a reporter plasmid, repression was rather observed with both GRH expression plasmids (Fig. 13B), probably because of squelching of the TFIID complex by GRH, because it is reported that GRH interacts with a component of the TFIID complex (33, 49). To further confirm the role of the GRH(UREF)-binding site as a target for GRH proteins, three GRH(UREF)-binding sites or their mutant derivatives were ligated upstream of the metallothionein basal promoter and used as a reporter plasmid. As shown in Fig. 14A, the three GRH(UREF)-binding sites stimulated the promoter activity 3-fold, probably because of endogenous GRH. Expression of the GRH(N) and GRH(O) further stimulated the promoter activity 62-fold (Fig. 14B) and 110-fold (Fig. 14C), respectively, whereas both proteins exerted only a marginal effect on the promoter carrying a mutant form of the GRH(UREF)-binding site. These results suggest that the GRH(UREF)-binding site is indeed a potential target for activation by GRH proteins. However, because the degree of activation of the PCNA promoter by transient expression of GRH was low, we can not exclude the possibility that GRH is not the major player in transcriptional regulation of the PCNA promoter in Kc cells. Further analysis is necessary to address this point.

DISCUSSION

The Drosophila PCNA gene promoter contains multiple regulatory elements including URE, DRE, E2F, and CFDD sites.
In the present study, we detected a protein factor, UREF, that binds to the region between 2130 and 2118 of the PCNA promoter. One-hybrid screening using UREF-binding site as a bait allowed cloning of a GRH cDNA. The nucleotide sequence required for binding to the GST-GRH fusion protein was indistinguishable from that for UREF, and a specific antibody to GRH reacted with UREF in embryo nuclear extracts. From these results we conclude that UREF is identical to GRH.

GRH is present in several tissues, where it appears to participate in different developmental programs. GRH is expressed in cuticle secreting cells during embryogenesis (32) and one of the putative target genes in these cells is the dopa decarboxylase (Ddc) gene, which is essential for cuticle formation (35, 50). GRH also appears to function as a transcriptional activator to regulate several other genes involved in epidermal development, including Ultrabithorax (Ubx), engrailed, and fushi tarazu (ftz) (33, 49, 51). GRH is also present in a particular set of cells in the embryonic and larval central nervous system (37, 52), although its target gene(s) in this site has yet to be identified. GRH may further function as a repressor, contributing to the mechanisms that restrict the expression of tailless and decapentaplegic to particular domains in embryo termini (53, 54). Although there is no indication so far of a role in cell cycle regulation or DNA replication, this is suggested by our present data.

The human α-globin transcription factor CP2 has been identified as a mammalian homologue of GRH/NTF-1 (52, 55, 56). CP2 is identical to LBP-1c/UBP-1, which binds to multiple sites within the human immunodeficiency virus long terminal repeat (57–59). CP2/LBP-1c/UBP-1 is also identical to the transcription factor LSF that specifically binds to and stimulates transcription from the SV40 major late promoter (60). Interestingly, LSF also binds to sites within the c-fos gene, ornithine decarboxylase gene, and thymidylate synthase gene promoters (61, 62). Activation of these cellular gene promoters and the SV40 late gene promoter is reported to be coupled with cell proliferation (63–66). It has also been noted that LSF is rapidly phosphorylated on mitogenic stimulation of resting T cells and its DNA binding activity is enhanced by this phosphorylation (61). However, the contribution of LSF-binding sites to activa-
tion of these cell cycle-regulated genes has yet to be determined. Our findings indicate that a GRH-binding site positively regulates the *Drosophila* PCNA gene, a DNA replication-related gene. The observation provides the first direct evidence for an involvement of GRH in regulation of DNA replication-related genes.

The nucleotide sequence required for binding to UREF (GRH) in the PCNA gene promoter was determined to be 5'-AAACCAGTTGGCA. This sequence matches 8 of 12 nucleotides of GRH-binding element 1 (be1, 5'-GAAACCAGTTAT) and 6 of 12 nucleotides of GRH-binding element 2 (be2, 5'-GAAACCGGTCTCT), respectively, in the Ddc gene (37). Sharing of nucleotides was also found between the binding site in the PCNA gene promoter and the NTF-1 (GRH)-binding consensus, 5'-(T/C)NAAC/C/TGGGT/T/C/T/C/GG examined with ftz, Ubx and Ddc genes (33). The element be1 functions in all GRH-expressing cells including the epidermis and the central nervous system, whereas the element be2 functions exclusively in the CNS (37). The higher similarity between the binding sites in the PCNA promoter and in be1 may reflect some function in wide variety of proliferating cells. In addition, the UREF (GRH) binding sequence in the PCNA promoter is also similar to the reported binding consensus sequence (5'-ANCACCGTGTNNCA) for the *Drosophila* snail gene product and its related proteins (67, 68). However, bacterially expressed Snail protein did not bind to the site in a band mobility shift assay (data not shown).

Although transgenic third instar larvae having a mutation in the UREF (GRH)-binding site (mutφ) had a reduced staining signal in the salivary glands, further reduction was observed with flies having a deletion to the position −119. The results thus indicate that the region upstream of the UREF (GRH)-binding site can stimulate PCNA promoter activity in the salivary glands. Other transcription factor(s) therefore might bind to an adjacent site upstream of the UREF (GRH)-binding site, although we have not succeeded in detecting such binding activity yet.

Acknowledgments—We thank Drs S. Bray for providing GRH cDNAs, S. Elledge for the AACT-*Drosophila* cDNA library, and M. Moore for comments on the English language used in the manuscript.

![Fig. 14. Effects of cotransfecting GRH expression plasmids on CAT activity directed by the metallothionein gene basal promoter carrying three copies of the wild type or mutant URE sequence.](https://www.jbc.org/content/268/14/2082.figure14.large)

**FIG. 14.** Effects of cotransfecting GRH expression plasmids on CAT activity directed by the metallothionein gene basal promoter carrying three copies of the wild type or mutant URE sequence. Aliquots of 2 μg of plasmids pTATACAT, p3UREwt-TATACAT, or p3UREmut-TATACAT were cotransfected into Kc cells with 0.05 μg of the pDhp70-L plasmid (A). 48 h after the transfection, cell extracts were prepared to determine the CAT expression levels and normalized to the luciferase activity. Averaged values obtained from four independent dishes with standard deviations are shown by bars as CAT activity relative to that of pTATACAT. Two μg of plasmid pTATACAT, p3UREwt-TATACAT, or p3UREmut-TATACAT was cotransfected into Kc cells with 0.05 μg of pDhp70-L plasmid and 2 μg each of pAct-GRH(N) (B) or pAct-GRH(O) (C). 48 h after the transfection, cell extracts were prepared to determine the CAT expression levels and normalized to the protein amount. Average stimulation (fold) of CAT activities by GRH expression plasmids are shown. Standard deviations between independent transfections are also shown.

| Plasmid       | Relative CAT activity (%) |
|---------------|---------------------------|
| pTATACAT      | 100                       |
| p3UREwt-TATACAT | 100                      |
| p3UREmut-TATACAT | 100                     |

| Plasmid       | Relative CAT activity (%) |
|---------------|---------------------------|
| 2 μg pTATACAT | 100                       |
| 2 μg p3UREwt-TATACAT | 100                    |
| 2 μg p3UREmut-TATACAT | 100                    |

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