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Transcriptional regulation of the *Drosophila-raf* proto-oncogene by the DNA replication-related element (DRE)/DRE-binding factor (DREF) system

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ABSTRACT

The DRE/DREF system plays an important role in transcription of DNA replication genes such as those encoding the 180 and 73 kDa subunits of DNA polymerase α as well as that for encoding PCNA. In this study, we found two sequences homologous to DRE (5′-TATCGATA-3′) in the 5′-flanking region (–370 to –357 with respect to the transcription initiation site) of the *D-raf* gene and confirmed transcriptional activity through gel mobility shift assays, transient CAT assays, and spatial patterns of *lac*Z expression in transgenic larval tissues carrying *D-raf* and *lac*Z fusion genes. Further, we demonstrated that the *D-raf* gene is another target of the Zerknüllt (Zen) protein with observation of *D-raf* repression by Zen protein in cultured cells and its ectopic expression in the dorsal region of the homozygous *zen* mutant embryo. The evidence of DRE/DREF involvement in regulation of the *D-raf* gene obtained in this study strongly supports the idea that the DRE/DREF system is responsible for the coordinated regulation of cell proliferation-related genes in *Drosophila*.

INTRODUCTION

Raf-1, a protein serine/threonine kinase located primarily in the cytosol (1,2), serves as central intermediate in many signaling pathways, ultimately regulating cell proliferation, differentiation, and development (3,4) by connecting upstream tyrosine kinases with downstream serine/threonine kinases such as mitogen-activated protein kinase (MAPK) and MAPK kinase (MAPKK) (3,5). A *Drosophila* homolog of the human *c-raf-1*, *D-raf* has been cloned and mutants defective for this gene have been identified (6–8). It has thereby been shown to be required for regulation of cell proliferation and differentiation (6,7,9,10). However, little is known about the control of *raf* proto-oncogene expression in *Drosophila*.

The DNA replication-related element (DRE) consisting of an 8 base pair (bp) palindrome, TATCGATA, is responsible for activating promoters of the *Drosophila melanogaster* PCNA (proliferating cell nuclear antigen) and DNA polymerase α-encoding genes, both in cultured cells (11) and in transgenic flies (12). Furthermore, a specific DRE-binding factor (DREF) consisting of an 80 kDa polypeptide homodimer has been purified (11), and a corresponding cDNA has recently been cloned (13).

Promoters of *Drosophila* DNA replication-related genes are repressed by the product of *zerknüllt* (*zen*) (14), a homeobox gene which regulates the differentiation of the optic lobe and the amnioserosa in the dorsal region of the *Drosophila* embryo (15–17). Repression of promoter activities by the Zen protein has been observed not only in cultured Kc cells but also in transgenic flies carrying the PCNA gene promoter-directed *lac*Z gene (14,18). Overexpression of Zen results in reduction of DREF activities in the cell (18). Therefore, DREF may be one of the key regulatory factors involved in proliferation- and differentiation-related control of DNA replication related genes (18).

In this study, we found two DRE-like sequences in the 5′-flanking region of the *D-raf* gene and have examined their role in promoter activity. The obtained results indicate that *D-raf*, which functions as a signal transducer, is indeed under the control of the DRE/DREF system, like DNA replication-related genes. We also report that the promoter activity of the *D-raf* gene is negatively regulated by the Zen protein, both in cultured cells and in living organisms.

MATERIALS AND METHODS

Plasmid constructions

A 1233 bp DNA fragment containing the *D-raf* promoter region (–878~+358 with respect to the transcription initiation site) (19) was isolated from plasmid pGEM-*Draf*4.3 bearing a genomic 4.3 kb *Bam*HI fragment (6) by digestion with *Bam*HI and *Pst*I. The fragment was blunt-ended with T4 DNA polymerase and subcloned

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into the *Sma*I site of pGEM-3. The direction of insert was examined by digestion with *Fok*I. The resultant plasmid was named pGEM-*Draf*1.23. The *D-raf* promoter region obtained from the plasmid pGEM-*Draf*1.23 by digestion with *Xba*I and *Sac*I was then inserted into the *Xba*I and *Sac*I sites of pSKCAT (14). The resultant plasmid was named p5′-878*Draf*CAT.

To construct a 2 bp insertional mutation, the plasmid pGEM-*Draf*1.23 was digested with *Cla*I targeting a site at the center of the *Draf*-DRE-like sequence. The digested DNA fragment was blunt-ended using T4 DNA polymerase and then self ligated with T4 DNA ligase. The resultant plasmid was confirmed to have an additional 2 bp GC sequence, ATCGCGAT, (inserted nucleotides underlined) by nucleotide sequencing. The mutant plasmid was digested with *Xba*I and *Sac*I, and then inserted into the *Xba*I and *Sac*I sites of pSKCAT. The resultant plasmid was named p5′-878In2*Draf*CAT.

The expression plasmid pAct5C-zen (20) contains *zen* cDNA placed under the control of the *Drosophila* actin 5C gene promoter (–2500 to +88) (21). The expression plasmid of mutant *zen*, pAct5C-zen-Δ1 contains an internal deletion from amino acids 137 to 236 of the Zen protein (20).

Oligonucleotides

All double-stranded oligonucleotides contained a 6 bp linker sequence recognizable by *Bgl*II and *Bam*HI and were chemically synthesized using a Applied Biosynthesis DNA Synthesizer. The *Draf*-DRE, *Draf*-DRE-mut1 and *Draf*-DRE-In2 oligonucleotides being as follows:

Draf-DRE
5′-gatccTTTATCGTTATCGATTGGTACAGCa-3′
3′-gAAATAGCAATAGCTAACCATGTCGtctag-5′

Draf-DRE-mut1
5′-gatccTTGCGCGTGCGCGATTGGTACAGCa-3′
3′-gAACGCGCACGCGCTAACCATGTCGtctag-5′

Draf-DRE-In2
5′-gatccTTTATCGTTATCGCGATTGGTACAGCa-3′
3′-gAAATAGCAATAGCGCTAACCATGTCGtctag-5′

where mutated bases are underlined and lower-case letters indicate the linker sequence. The double-stranded 30 bp oligonucleotides for *Draf*-DRE contain the 24 bp DRE like-containing sequence of the *D-raf* gene promoter and the 6 bp linker sequence, while the *Draf*-DRE-mut1 contains two 3 bp substitutions in this DRE sequence. *Draf*-DRE-In2, a double-stranded 32 bp oligonucleotide, contains a 2 bp insertion in the DRE sequences of *Draf*-DRE. Control double-stranded oligonucleotides, DRE-P and DRE-PM, being as follows:

DRE-P
5′-gatccCTGCCTGCTATCGATAGATTCAGGa-3′
3′-gGACGGACGATAGCTATCTAAGTCCtctag-5′

DRE-PM
5′-gatccCTGCCTGCTTACGATAGATTCAGGa-3′
3′-gGACGGACGAATGCTATCTAAGTCCtctag-5′
were also generated as in Hirose *et al.* (11).

Gel mobility shift analysis

The gel mobility shift analysis was performed as reported previously (11). Kc cell nuclear extracts and *Escherichia coli* lysates containing GST-DREF(16-608) fusion protein were prepared as described elsewhere (13). These were then added to reaction mixtures containing 15 mM HEPES (pH 7.6), 60 mM KCl, 0.1 mM EDTA, 1 mM DTT, 12% glycerol, 0.5 µg of sonicated calf thymus DNA (average size of 0.2 kb) and double-stranded ^{32}P-labeled synthetic oligonucleotides (10 000 c.p.m.) and incubated for 15 min on ice. For this step, unlabeled DNA fragments were added as competitors. DNA–protein complexes were electrophoretically resolved on 6% nondenaturing polyacrylamide gels in 50 mM Tris/borate (pH 8.3), 1 mM EDTA and 2% glycerol at 25°C. Gels were dried and autoradiographed. The gel shift assay was also performed with anti-DREF monoclonal antibody No. 1 and anti-DREF monoclonal antibody No. 4 (13). Kc cell nuclear extracts were mixed with each antibody, incubated for 2 h on ice, added to mixtures containing ^{32}P-labeled synthetic oligonucleotides (10 000 c.p.m.) and 0.5 µg poly(dI–dC), and then incubated for 15 min on ice as described above.

Cell culture, DNA transfection and CAT assay

Drosophila Kc cells (22) were grown at 25°C in M3 (BF) medium (Sigma) supplemented with 2% fetal bovine serum and 0.5% penicillin–streptomycin (GIBCO-BRL). Kc cells (5×10^6/dish) were plated into 60 mm plastic dishes 24 h before DNA transfection by the calcium phosphate coprecipitation method, as described elsewhere (23). Each transfection also included 10 µg of the *D-raf* gene promoter-CAT plasmid as a reporter. For cotransfecting Zen expression plasmids, unless otherwise specified, 2 µg of p5′-878*Draf*CAT or 0.5 µg of p5′-168DPCNACAT and 1–8 µg of expression plasmid were transfected. The total amount of DNA for transfection was adjusted to 10 µg/dish with pGEM-3 plasmid DNA. Cells were harvested at 48 h after DNA transfection. Cell extracts for determination of CAT activites were prepared as described (24). The spots corresponding to acetylated [^{14}C]chloramphenicols were taken from thin layer plates and radioactivities counted in a toluene-based scintillator. CAT activities were normalized to protein amounts, determined using a BioRad protein assay kit (25).

Germ line transformation and analysis of expression patterns

P-element-mediated transformation and establishment of homozygous transformant stocks were performed as described previously (26,27). Three independent transformant lines were established for p5′-878*Draf*lacZ (28).

Expression patterns for *lac*Z were analyzed by X-gal staining as described earlier (29). Larval tissues were dissected, immersed in fixative (12 mM sodium cacodylate buffer, pH 7.3/26% glutaraldehyde) for 15 min at room temperature, and then incubated with a staining solution containing 0.2% X-gal in the dark at 37°C for 5–16 h.

Whole-mount *in situ* hybridization for detecting expression of endogenous *D-raf* gene in wild-type *Drosophila* and the homozygous *zen*^{w36} mutant (30) was conducted essentially as described by Tautz and Pfeifle (31). Embryos were collected, aged at 25°C, dechorinated and fixed, then were stored in 70% ethanol at –70°C and rehydrated when needed. As a probe, The 2.3 kb *Hin*cII–*Sma*I fragment from plasmid pGEM-*Draf*4.3 was labeled by random priming with a Digoxigenin Non-radioactive DNA Labeling and Detection Kit (Boeringer Mannheim). Embryos were developmentally staged using criteria described by Campos-Ortega and Hartenstein (17).

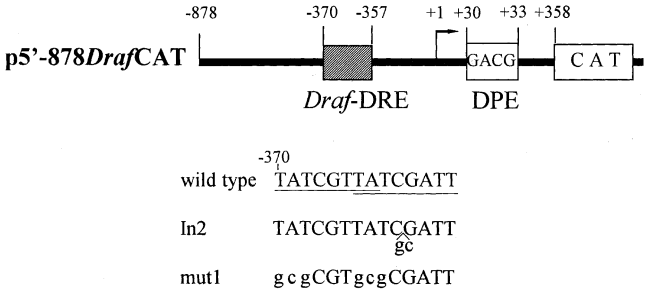

Figure 1. DRE-like sequences of the *D-raf* gene. The construct of the wild-type *Draf*-CAT fusion gene (p5′-878*Draf*CAT) is shown. The *D-raf* promoter (*Draf*-DRE) contains an overlapping pair of DRE-like sequences as indicated by the shaded box. The locations of sites relative to the transcription initiation site are indicated by the numbers with vertical lines. Insertions or base substitutions in the wild-type sequences are shown by lower case letters. The conserved downstream basal promoter element (DPE), which is present in *Drosophila* TATA-box-deficient promoters, is shown in the +30 to +33 region downstream of the transcription initiation site of the *D-raf* promoter.

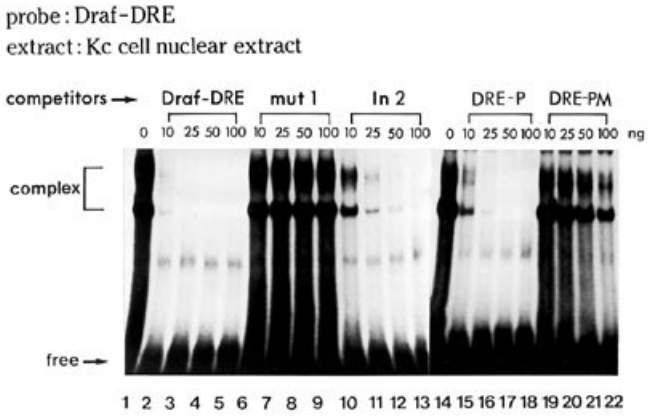

Figure 2. Competition for complex formation between *Draf*-DRE oligonucleotides and Kc cell nuclear extracts. ^{32}P-labeled double-stranded *Draf*-DRE oligonucleotides were incubated with Kc cell nuclear extract in the presence of the indicated competitor oligonucleotides. *Draf*-DRE, oligonucleotide containing the wild-type *Draf*-DRE sequence; mut1, oligonucleotide containing multiple base substitutions in the *Draf*-DRE sequence *Draf*-DRE-mut1; In2, oligonucleotide containing 2 bp insertional mutation in the *Draf*-DRE sequence *Draf*-DRE-In2; DRE-P, oligonucleotide containing the DRE sequence from the *Drosophila* PCNA gene; DRE-PM, the DRE-P oligonucleotide having mutations in the DRE sequence.

RESULTS

The DRE-like sequences located in the promoter region of the *D-raf* gene

In the 5′-flanking region of the *D-raf* gene, we found two adjoining sequences homologous to DRE (5′-TATCGATA-3′) TATCGTTATCGATT, *Draf*-DRE extending from positions –370 to –357 with respect to the transcription initiation signal site (32–34) of the *D-raf* gene (6,8,10) (Fig. 1). Each of these sequences matches 7 bp out of the 8 bp DRE sequence. We also found a conserved downstream basal promoter element (DPE) consensus sequence (19) in the +30 to +33 region downstream of the transcription initiation site of the *D-raf* promoter (Fig. 1). The DPE consensus sequence is usually located ~30 nucleotides downstream of the RNA start site of the *Drosophila* TATA-box-deficient (TATA-less) promoters (19).

In this study, to investigate activity of *D-raf* gene promoter in cultured cells, we constructed the reporter plasmid p5′-878*Draf*CAT, in which the upstream region fragment (–878 to +358 with respect to the transcription initiation site) of the *D-raf* gene was placed adjacent upstream of the CAT gene (Fig. 1). We previously found that the promoter region is sufficient for endogenous expression of the *D-raf* gene (28).

DREF binding to the DRE-like sequences in the *D-raf* promoter

For confirmation of the role of the DRE/DREF system in transcription of the *D-raf* gene, we examined whether *Draf*-DRE sequences can be recognized by DREF, the DRE-binding factor identified previously (11). Gel mobility shift assays were thus performed using Kc cell nuclear extracts as the control source of DREF. Specific DNA–protein complexes could thereby be detected using a chemically synthesized oligonucleotide carrying the *Draf*-DRE sequence as a probe (Fig. 2, lanes 1 and 14). Two shifted bands on the gel suggest that there are at least two complexes, although it is not clear yet if these reflect occupation of one site and both sites. The complex with ^{32}P-labeled *Draf*-DRE was diminished by adding excess amounts of unlabeled *Draf*-DRE (Fig. 2, lanes 2–5) or DRE-P (Fig. 2, lanes 15–18), an oligonucleotide containing the DRE sequence from the *Drosophila* PCNA gene, as a competitor (11). However, *Draf*-DRE-mut1 carrying the multi-base-substitution inside the DRE sequence (Fig. 2, lanes 6–9) and DRE-PM (Fig. 2, lanes 19–22) did not diminish the complex formation. *Draf*-DRE-In2 carrying the 2 bp insertion more or less diminished the complex formation (Fig. 2, lanes 10–13), suggesting that the DRE-related sequence TATCGTTA, can also function as DRE.

Gel mobility shift assays were also carried out with an extract of *E.coli* producing GST-DREF(16-608) fusion protein and [^{32}P]*Draf*-DRE. DNA–protein complex was detected (Fig. 3, lanes 1 and 14). Specificity of binding was evident in competition with DRE-P, DRE-PM, *Draf*-DRE, *Draf*-DRE-mut1 and *Draf*-DRE-In2. The oligonucleotide *Draf*-DRE effectively competed for the binding (Fig. 3, lanes 2–5). The oligonucleotides DRE-P carrying one DRE sequence and *Draf*-DRE-In2 carrying 2 bp insertion in one of the two adjoining DRE-like sequences less efficiently competed for the binding (Fig. 3, lanes 10–13 and 15–18).

In contrast, the oligonucleotides DRE-PM and *Draf*-DRE-mut1 carrying multiple base substitutions did not compete at all (Fig. 3, lanes 6–9 and 19–22).

Examination of the effects of the addition of anti-DREF monoclonal antibodies on DNA–protein complex formation revealed reduction with antibody No. 1 (Fig. 4, lanes 1–3) and a super-shifted with antibody No. 4 (Fig. 4, lanes 5–7). These results thus clearly indicate that a factor containing DREF or DREF itself can bind to the DRE-like sequences of the *D-raf* promoter.

To test whether DRE-related sequences are necessary for activation of the promoter of the *D-raf* gene, we introduced a 2 bp insertional mutation into the sequence of the reporter plasmid p5′-878*Draf*CAT. The mutation led to an extensive reduction of CAT activity (Fig. 5).

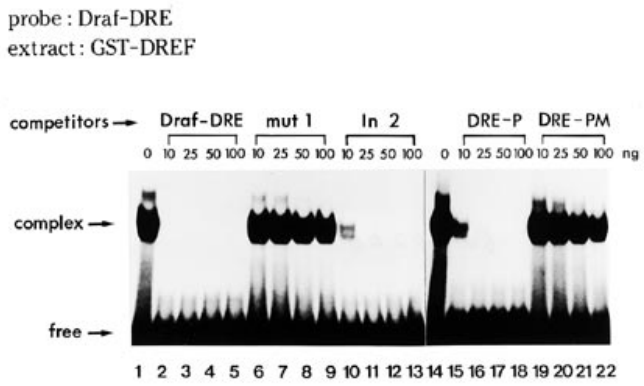

Figure 3. Binding of DREF to the *Draf*-DRE sequence. ^{32}P-labeled *Draf*-DRE oligonucleotides were incubated with an extract of *E.coli* producing GST-DREF(16-608) fusion protein in the presence of the indicated competitor oligonucleotides. *Draf*-DRE, oligonucleotide containing the wild-type *Draf*-DRE sequence; mut1, oligonucleotide containing multiple base substitutions in the *Draf*-DRE sequence *Draf*-DRE-mut1; In2, oligonucleotide containing 2 bp insertional mutation in the *Draf*-DRE sequence *Draf*-DRE-In2; DRE-P, oligonucleotide containing the DRE sequence from the *Drosophila* PCNA gene; DRE-PM, the DRE-P oligonucleotide having mutations in the DRE sequence.

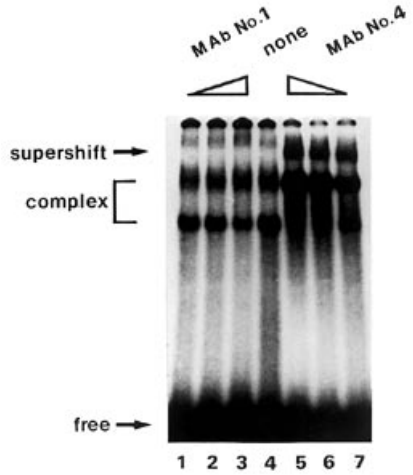

Figure 4. Effects of antibodies on DRE–DREF complex formation. ^{32}P-labeled *Draf*-DRE oligonucleotides were incubated with Kc cell nuclear extracts in the absence (lane 4) or presence (lanes 1–3 and 5–7) of anti-DREF monoclonal antibody No. 1 (0.2, 0.4 and 0.8 µl of culture supernatant) and anti-DREF monoclonal antibody No. 4 (0.8, 0.4 and 0.2 µl of culture supernatant). MAb No. 1, anti-DREF monoclonal antibody No. 1; MAb No. 4, anti-DREF monoclonal antibody No. 4.

Spatial patterns of *lac*Z expression in the salivary glands, the brain lobes and the imaginal discs from transgenic flies carrying *D-raf* (–878 to +525 with respect to the transcription initiation site) and the *lac*Z fusion gene was earlier detected (28). In the salivary glands of third instar larvae, *Draf-lac*Z expression was only detected in the imaginal rings (28), consistent with a phenotypic defect in the salivary gland of the *D-raf* mutant leading to smaller numbers of imaginal salivary gland cells than in normal larvae (6). This same pattern of immunochemical localization of DREF and PCNA was found for the salivary glands of third instar larvae (35). These results suggest that DREF

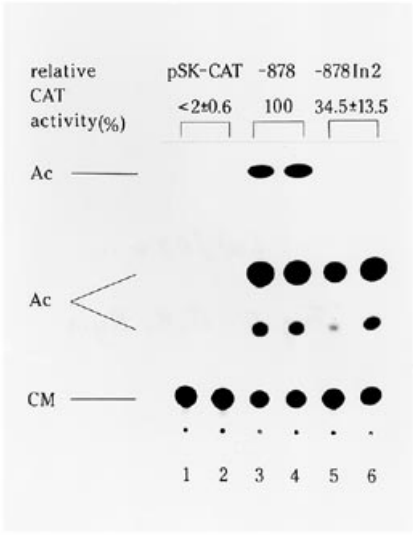

Figure 5. Requirement of the *Draf*-DRE sequence for expression of the *D-raf* gene. CAT plasmids harboring wild-type or mutant *D-raf* promoter were transfected into Kc cells, and after 48 h extracts were prepared to determine the CAT expression levels. Values were normalized to protein amounts. Average values obtained from four independent dishes with standard deviations are given as CAT activity relative to that of the wild-type plasmid p5′-878*Draf*CAT. Acetylated forms of [^{14}C]chloramphenicol were undetectable in the promoter-less CAT (pSKCAT) plasmids included as controls (lanes 1 and 2). Acetylated and nonacetylated forms of [^{14}C]chloramphenicol are marked by Ac and CM, respectively. The sets of two adjacent lanes represent duplicate independent transfections. –878, p5′-878*Draf*CAT; –878In2, p5′-878In2*Draf*CAT.

may regulate the expression of the *D-raf* gene as well as DNA replication-related genes in these larval tissues.

Repression of *D-raf* promoter-directed CAT expression by the Zerknüllt homeodomain protein

Promoters of *Drosophila* DNA replication-related genes are repressed by the product of the *zen* gene (12,18). Whether Zen protein can affect transcription of the *D-raf* gene was therefore examined in cultured cells and in living organisms.

In cultured cells, cotransfection assays were carried out with the plasmid p5′-878*Draf*CAT and plasmids bearing wild-type or mutant Zen under the direction of the *Drosophila* actin 5C promoter (21), which is highly active in *Drosophila* cells. As a control, the plasmid p5′-168DPCNACAT carrying the upstream region (–168 to +24) of the PCNA gene was cotransfected with wild-type or mutant Zen expression plasmids.

Wild-type Zen repressed the activity of the *D-raf* gene promoter, with the degree of decrease being progressively augmented by increasing the amount of effector plasmid (Fig. 6A). The plasmid pAct5C-zen-Δ1 carrying an internal in frame deletion (99 amino acid residues including 13 carboxyl-terminal amino acid residues of the homeobox) only slightly affected the CAT expression by p5′-878*Draf*CAT or p5′-168DPCNACAT (Fig. 6A and B). The results obtained indicate that the active Zen protein can specifically repress the *D-raf* promoter activity, as was the case with the PCNA gene promoter as well as the DNA polymerase α promoter (14,18). The extent of repression of the *D-raf* promoter activity by Zen protein was similar to that of the PCNA promoter activity (Fig. 6A and B).

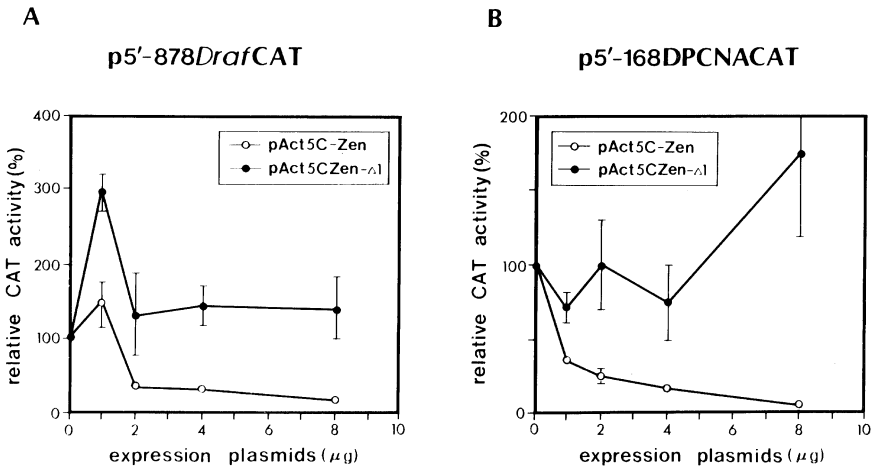

Figure 6. Effect of cotransfecting Zen expression plasmids on CAT expression directed by the regulatory region of the *D-raf* gene or the *Drosophila* PCNA gene. Two micrograms of plasmid p5′-878*Draf*CAT(A) were cotransfected into Kc cells with expression plasmids pAct5C-zen or pAct5C-zenΔ1. Half micrograms of plasmid p5′-168DPCNACAT(B) were cotransfected into Kc cells with expression plasmids pAct5C-zen or pAct5C-zenΔ1, as a control. The total amount of DNA for transfection was adjusted to 10 μg/dish with pGEM-3 plasmid DNA. At 48 h after transfection, cell extracts were prepared to measure CAT activity. The CAT sensitivities were quantified and plotted against activity in the absence of effector plasmid. The relative values are averages of results from three independent transfections.

In embryos with the homozygous *zen* mutant genotype (30), ectopic expression of PCNA was earlier detected in the abnormally expanded dorsal region (14). We therefore examined the spatial patterns of *D-raf* transcripts in wild-type embryos and embryos with the homozygous *zen* mutation by *in situ* hybridization. As expected, *D-raf* transcripts were not detected in the dorsal region of the wild-type embryos, in the area where the *zen* gene was expressed, but ectopic expression did occur in the same region of the homozygous *zen* mutant embryos (Fig. 7).

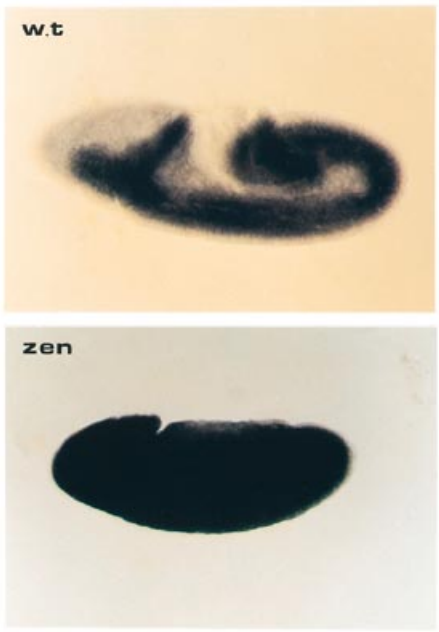

Figure 7. Ectopic expression of the *D-raf* gene in the dorsal region of embryos homozygous for the *zen* mutation. Expression patterns of endogenous *D-raf* in wild-type (w.t) and homozygous zen^{w36} mutant (*zen*) embryos were detected by *in situ* hybridization. The embryos shown were at early stage 9, the anterior to the left and the dorsal aspect facing upward. Ectopic expression of the *D-raf* gene in the dorsal region of the homozygous zen^{w36} mutant embryo is apparent.

DISCUSSION

Drosophila DRE/DREF system plays an important role in the regulation of DNA replication-related genes such as those encoding the 180 kDa (11) and 73 kDa (36) subunits of DNA polymerase α, PCNA (37) and cyclin A (38). *D-raf* has been demonstrated to bear multiple functions in the regulation of both proliferation and differentiation of cells during development (6–8). It is expressed throughout development in a wide range of tissues with higher levels of expression in the ovary and in tissues containing rapidly proliferating cells (6,28). Multiple regulatory elements should participate in the expression of *D-raf*, and here we have demonstrated that DRE is one of them.

Two overlapping DRE-like sequences are found in the 5′-flanking region (–370 to –357 with respect to the putative transcription initiation site) of *D-raf* (Fig. 1). A gel mobility shift assay using Kc cell extracts and bacterially produced GST-DREF fusion protein clearly demonstrated that the sequences are indeed the target for the binding of DREF (Figs 2, 3 and 4). Disruption of one of the DRE elements results in a significant reduction of CAT activity in the cells transiently expressing the *CAT* gene fused to the 5′-flanking sequence of *D-raf* (Fig. 5). These observations strongly suggest that the expression of *D-raf* is under the control of the DRE/DREF system as are the DNA replication-related genes. A reporter *lacZ* gene fused to the 5′-flanking sequence of *D-raf* (–878 to +525 with respect to the transcription initiation site) is expressed in the tissues containing proliferating cells such as the imaginal rings of the salivary glands and the imaginal discs (28). This indicates that *D-raf* is expressed at higher levels in tissues with rapidly proliferating cells and that the DRE/DREF system would be for the activation.

It has been demonstrated that the transcription from the gene for PCNA is repressed by Zerknüllt in the embryonic dorsal region

including the amnioserosa (14). Although the precise mechanism for this repression remains to be elucidated, it has been demonstrated that the DRE sequence in the 5′-flanking region of the *PCNA* gene is responsible for the repression and that Zerknüllt may affect the amount or activity of DREF (14). *D-raf* was demonstrated to be similarly under the regulation of Zerknüllt both *in vitro* (Fig. 6) and *in vivo* (Fig. 7). These observations further support the idea that the expression of *D-raf* is under the control of the DRE-DREF system in concert with the DNA replication-related genes.

The major role of *D-raf* in proliferation would be the transduction of transmembrane growth-stimulating signals into the nucleus in the G_0/G_1 transition and G_1 phase as has been demonstrated with mammalian Raf-1 (39,40), and the activation of the DRE/DREF system should follow this signaling process. Then, what is the significance of the coordinated expression of *D-raf* with the DNA replication-related genes? There is no evidence for the participation of *D-raf* in DNA replication, but it has been reported that Raf-1 is activated during M phase (41). On the other hand, we have observed no significant accumulation of neuroblast cells arrested in M phase in temperature-sensitive mutant larvae of *D-raf* at non-permissive temperature (7), and no abberation of mitosis in the cleavage division stage embryos lacking both maternal and zygotic *D-raf* (L. Tsuda, H.-Y. Ha and Y. Nishida, manuscript in preparation). Thus, it is possible that *D-raf* may function both in G_1 and M phase and its redundant function in M phase is dispensable. Expressions of DNA polymerase α, PCNA and cyclin A are regulated by the cell cycle-dependent transcription and degradation of their proteins (42,43). In contrast, *D-raf* seems to be quite stable, since the maternally-provided *D-raf* is sufficient to support the development of animals hemi- or homozygous for the null functional mutation of *D-raf* until late third instar larval or early pupal stages (6,8). The DRE/DREF system-dependent expression of *D-raf* may result in a persistent increase of *D-raf* in the progenitor cells, and this would elevate their competence to growth-stimulating signals allowing their rapid and continuous proliferation as observed in the imaginal discs. It is of interest to learn whether the expression of *Dsor1* and *rolled (rl)* encoding the homologs of MAP kinase kinase and MAP kinase, respectively (44,45), are also under the control of the DRE/DREF system.

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