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Transcriptional Inhibition of the *Drosophila raf* and PCNA Genes by the Homeodomain Protein Ftz and Engrailed

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Raf-1 is a key molecule in many signaling pathways and is required for the regulation of cell proliferation and differentiation. It is also related to the tumorigenesis in some cancer cells. And proliferating cell nuclear antigen (PCNA) is required for the cellular DNA synthesis and cell cycle progression as an accessory protein of DNA polymerase δ. We have found that in the promoter region of *D-raf, Drosophila* homolog of human *c-raf-1*, there exist several putative homeodomain protein binding sites. Because in general, homeodomain proteins serve as transcription factors and regulate the expression of affected genes, we investigated the effect of homeodomain proteins on the expression of *Drosophila raf* and PCNA genes. Although the promoter region of PCNA gene used in this study has no consensus sites for the homeodomain protein binding, both genes were repressed by the homeodomain proteins, Ftz (Fushi tarazu) and Engrailed in cotransfection assays. These results suggest the possibility that the homeodomain proteins, Ftz and Engrailed, may inhibit cell proliferation through transcriptional inhibition of cell proliferation-related genes such as *raf* and PCNA.

Key Words: *Drosophila, Raf*, PCNA, Ftz, Engrailed, Cotransfection assay

INTRODUCTION

Raf-1, a protein serine/threonine kinase located primarily in the cytosol, has been highlighted as a potential secondary signal transducer, and may be played a critical role in the control of proliferation and differentiation.[19,28,13] The Raf-1 serves as a central intermediate in many signaling pathways, ultimately regulating cell proliferation, differentiation, and development[4,6] by connecting upstream tyrosine kinase with downstream serine/threonine kinases such as mitogen-activated protein kinase (MAPK) and MAPK kinase (MAPKK).[4,29] Although mammals carry three genes c-*raf, A-raf* and *B-raf*, as a gene family, *Drosophila* carries only a single *raf* gene called *D-raf*.[24]

The proliferating-cell nuclear antigen (PCNA),[23] also known as cyclin, is a nuclear protein, the expression of which correlates with the proliferating state of the cells.[21,34] PCNA is an auxiliary protein for DNA polymerase δ [3] and is one of the essential factors for synthesis of the leading strand in the replication of simian virus 40 DNA.[26,27] This protein is also important for cellular DNA synthesis and cell cycle progression.[15] The cDNAs and genes for

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PCNA from mammals,[1,22,39] *Drosophila,*[39] plants[33] and yeast[2] have been cloned and completely sequenced.

Pattern formation in *Drosophila* embryos appears to be guided mainly by regulator of transcription. One class of transcriptional regulator, the homeodomain-containing proteins, make up ~50% of the gene products that genetics has implicated in patterning.[18,31] The homeodomain is a highly conserved sequence motif often found in proteins,[31] a number of homeodomain proteins bind to DNA with similar sequence specificity.[7,14,18] Many homeodomains recognize similar DNA sites having the common core sequence TAAT.[11] The *fushi-tarazu* and *engrailed* genes encode homeodomain proteins Ftz and Engrailed, respectively. These proteins act as a transcriptional regulators with the DNA binding being mediated by the homeodomain. Ftz is a zygotically expressed factor involved in the control of embryonic segmental pattern formation. Engrailed plays a critical role in the generation and maintenance of parasegments, the fundamental units of the insect body plan.[8,20,35]

It is essential for tumorigenesis that genes for cell cycle progression are constitutively expressed. Thus Raf and PCNA proteins may both implicated in the tumor progression. Actually, the expression level of *raf* and PCNA genes has been reported to be increased in some cancer cells.[16,17,25,36] Particularly, c-Raf can also protect tumor cells from undergoing programmed cell death.[17] Therefore, the interference with expression or function of these genes could represent a powerful means for improving the efficiency of anti-cancer therapy.

We found several putative homeodomain protein binding sites in the promoter region of the *Drosophila raf* and PCNA genes. In this work, we investigated the effect of homeodomain proteins, Ftz and Engrailed, on the expression of *Drosophila raf* and PCNA genes in *Drosophila* Kc cultured cells.

MATERIALS AND METHODS

1) Plasmids

The plasmid p5'-168DPCNACAT contains the *Drosophila* PCNA gene fragment spanning from −168 to +23 placed upstream of the chloramphenicol acetyltransferase (CAT) gene in the plasmid pSKCAT.[38] The plasmid p5'-663*D-raf*CAT was constructed previously.[30] Briefly, a 1233-bp DNA fragment containing the −663 to +573 region (with respect to the transcription initiation site) of the *Drosophila raf* gene was cloned into the *Xba*I and *Sac*I sites of pSKCAT.

The expression plasmid pAct5C-*en* and pAct5C-*ftz* contain full-length *engrailed* and *fushi tarazu* cDNA placed under the control of the *Drosophila* actin 5C promoter (−2500 to +88).

2) Cell culture, DNA transfection, and Chloramphenicol acetyltransferase (CAT) assay

Drosophila melanogaster embryonic Kc cells[10] were grown at 25°C in M3 (BF) medium[5] (Sigma) supplemented with 2% fetal bovine serum (FBS) inactivated at 56°C for 45 min and 0.5% Penicilin-Streptomycin (PS, GIBCO BRL).

Cells were plated at about 5×10^6 cells per 60 mm dish at 16 h before DNA transfection. DNA was transfected into cells by the calcium phosphate coprecipitation technique described elsewhere.[9] Each transfection contained 2 μg of p5'-168DPCNACAT or p5'-663*D-raf*CAT as reporters and 2 μg, 5 μg or 10 μg of pAct5C-*en* or pAct5C-*ftz* as effectors. Total amount of DNA was maintained to 12 μg by adding pUC18.

Cells were harvested at 48 h after transfection. Cell extracts were prepared, and the CAT activities were measured as described previously.[37] The radioactivities of acetylated chloramphenicol on thin layer plates were quantified with an imaging analyzer BAS1500 (Fuji Film). Transfections were performed at least three times in duplicate.

RESULTS

Repression of PCNA promoter-directed CAT expression by the Ftz and Engrailed homeodomain proteins

Drosophila fushi-tarazu and *engrailed* genes encode for nuclear protein with a homeodomain.

This homeodomain has been shown to mediate Ftz and Engrailed binding by interacting with specific bases and phosphate groups through major and minor grooves in the DNA. However, divergent homeoproteins including Ftz and Engrailed can recognize similar DNA sequences.[12]

To determine whether the homeodomain proteins, Ftz and Engrailed, can affect the transcription of the PCNA gene, a cotransfection assay using *Drosophila* embryonic Kc cells was carried out. Plasmid p5'-168DPCNACAT, carring the 5' upstream region (−168 to +23) of the PCNA gene linked to the CAT-coding region, was used as the reporter plasmid. The expression plasmids for Ftz, pAct5C-*ftz*, and that for Engrailed, pAct5C-*en*, were used as the effector plasmid (Fig. 1). Expressions of the Ftz and of the Engrailed are directed by the *Drosophila* actin 5C promoter, which is highly active in *Drosophila* cells.

The CAT activity of the plasmid p5'-168DPCNACAT was reduced by overexpressed Ftz, as shown in Fig. 2A. The Engrailed protein also repressed the PCNA promoter-directed CAT expression (Fig. 2B). The extent of repression seems to be

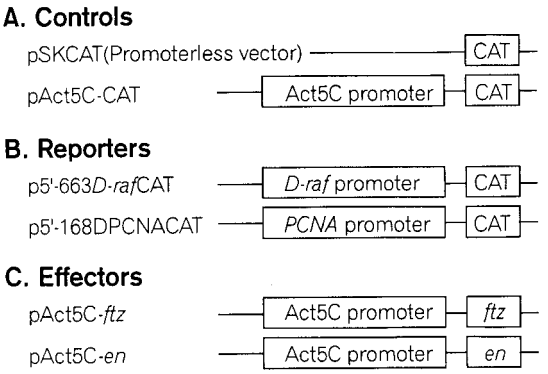

Fig. 1. The constructs of plasmids used for cotransfection assays. The plasmids pSKCAT and pAct5C-CAT were used as a negative and positive control, respectively. The plasmid p5'-168DPCNACAT contains the PCNA gene fragment spanning from −168 to +23 with respect to the transcription initiation sites placed upstream of the CAT gene in the plasmid pSKCAT. The plasmid p5'-663*D-raf*CAT contains the *D-raf* gene fragment spanning from −663 to +573 with respect to the transcription initiation sites placed upstream of the CAT gene in the plasmid pSKCAT. The expression plasmid pAct5C-*ftz* and pAct5C-*en* contains full-length *ftz* and *en* cDNA placed under the control of the *Drosophila* actin 5C promoter (−2500 to +88). The CAT activities of the plasmid, pAct5C-CAT, were always very higher than that of the plasmids, p5'-168DPCNACAT or p5'-663*D-raf*CAT (data not shown).

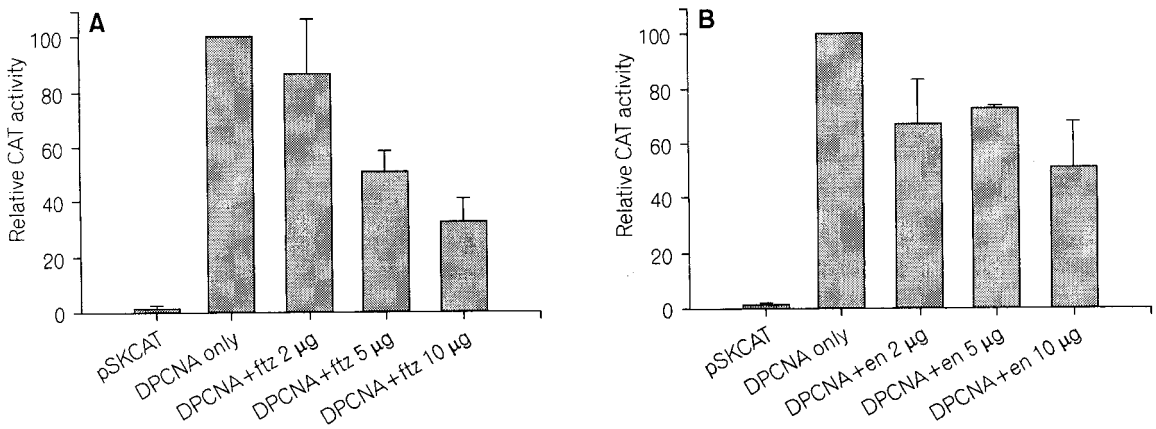

Fig. 2. Expression of the p5'-168DPCNACAT fusion gene cotransfected with *fushi tarazu* or *engrailed* expression vectors in *Drosophila* Kc cells. Each transfection included 2 μg of p5'-168DPCNACAT plasmid and 2 μg, 5 μg, or 10 μg of pAct5C-*ftz* (A) or pAct5C-*en* (B) expression vector, respectively. The total amount of DNA for transfection was adjusted to 12 μg/dish with pUC18. 48 h after transfection, cell extracts were prepared to determin the CAT expression levels. CAT activities were normalized to protein amounts measured with the BCA Protein Assay Reagents (PIERCE) and quantified with BAS 1500 Image Analyzer (Fuji Film). The promoterless vector, pSKCAT was used as a negative control. Results were obtained from at least three independent experiments.

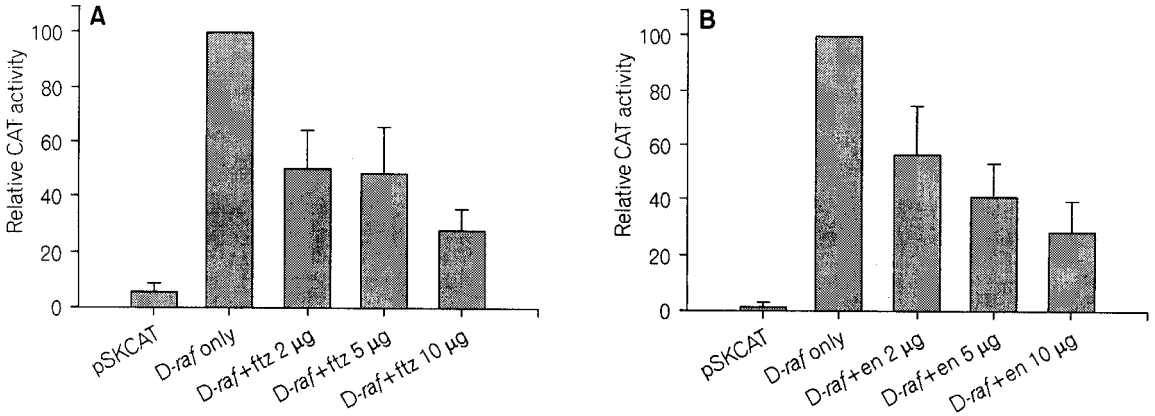

Fig. 3. Expression of the p5'-663*D-raf*CAT fusion gene cotransfected with *fushi tarazu* or *engrailed* expression vectors in *Drosophila* Kc cells. Each transfection included 2 μg of p5'-663*D-raf*CAT plasmid and 2 μg, 5 μg, or 10 μg of pAct5C-*ftz* (A) or pAct5C-*en* (B) expression vector, respectively. The total amount of DNA for transfection was adjusted to 12 μg/dish with pUC18. 48 h after transfection, cell extracts were prepared to determin the CAT expression levels. CAT activities were normalized to protein amounts measured with the BCA Protein Assay Reagents (PIERCE) and quantified with BAS 1500 Image Analyzer (Fuji Film). The promoterless vector, pSKCAT was used as a negative control. Results were obtained from at least three independent experiments.

somewhat dependent on the amount of effectors used. However, when cotransfected with 10 μg of pAct5C-*ftz* or pAct5C-*en*, Ftz repressed the promoter activity by up to 30%, but Engrailed only to 50%. This suggests Ftz could repress the activity of PCNA gene stronger than that of Engrailed.

Repression of *D-raf* promoter-directed CAT expression by the Ftz and Engrailed homeodomain proteins.

We next examined the effects of Ftz and Engrailed on the expression of *Drosophila raf* gene. In the promoter region of the gene, clustered sequences similar to the binding sites for *Drosophila* homeodomain proteins are located in the 5'-flanking region. To test whether the Ftz and Engrailed homeodomain proteins can actually affect the transcription of the *D-raf* gene, Kc cells were cotransfected by the calcium phosphate method, using the reporter plasmid p5'-663*D-raf*CAT carrying the 5' upstream region (−663 to +573) of the *D-raf* gene linked to the CAT-coding region, and the effector plasmids pAct5C-*ftz* and pAct5C-*en*. The CAT activity of the plasmid p5'-663*D-raf*CAT was also reduced by both the overexpressed Ftz and Engrailed, and the pattern of repression was similar to that of DPCNA gene (Fig. 3A and B). Unlike the case of PCNA gene, the extent of repression of *Drosophila raf* gene by Ftz or Engrailed was nearly identical. Both homeodomain proteins could repress the expression of *D-raf* gene near to 30% when cotransfected with 10 μg of pAct5C-*ftz* or pAct5C-*en*.

DISCUSSION

The homeobox, a 180 nucleotide DNA sequences, was discovered during the cloning and sequencing of the homeotic selector genes.[28] This DNA sequences encodes an amino acid sequences called the homeodomain and it was also found in other genes that control *Drosophila* development, such as *bicoid, even-skipped, engrailed* and *fushi tarazu*. The homeodomain is a DNA-binding domain, and the proteins containing the homeodomain are thought to act as transcription factors.[6]

The presence of clusters of 10-bp sequences similar to the binding sites for *Drosophila* homeodomain proteins was noted in the regions from nucleotide position −165 to −357 of the *Drosophila* PCNA gene.[39] And we have found several putative homeodomain protein binding sites in the regions from nucleotide position −489 to −433 of the *Drosophila raf* gene. We thought the possibility

that the expression of the *Drosophila raf* and PCNA genes may be under the control of genes coding for homeodomain proteins.

By transient expression assay, we examined the effect of *Drosophila* homeodomain proteins, Ftz and Engrailed, on the expression of the *Drosophila raf* and PCNA genes. The promoter activities of both genes were reduced when cotransfected with Ftz or Engrailed expression plasmids in a somewhat concentration-dependent manner. And the extent of repression by Ftz was shown to be rather stronger than that of Engrailed in the case of PCNA gene. However, the construct of PCNA promoter used in this study only has up to −168 position. Nevertheless, the promoter activity was affected by Ftz or Engrailed. This shows that the DPCNA gene may be controlled indirectly by homeodomain proteins in addition to the possibility that it is under the direct control of homeodomain proteins.

As the PCNA and *raf* genes are both necessary to the maintenance of proliferative state of cells, to the progression of tumors, and to the maintenance of tumor cells by protecting from apoptosis,[16,17,25,32,36] our results also suggest the possibility that cells in proliferative state including cancer cells may be down-regulated by homeodomain proteins, Ftz and Engrailed that these homeodomain proteins could provide an effective way to the treatment of cancer.

In order to investigate the possibility, first, the homeodomain-affected promoter region of *Drosophila raf* and PCNA genes should be identified in further study.

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