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Analysis of the Role of STAT Binding Site in the *Drosophila raf* Promoter Region Using Transgenic Flies

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Abstract

STATs activated by various cytokine and growth factors trigger a quick response in the nucleus and induce changes in gene expression. We have found the sequences homologous to STAT binding site in the 5′-flanking region of the *D-raf* gene. In this study, we examined role of the STAT binding site in *D-raf* gene promoter activity *in vivo* by using transgenic flies. The reporter plasmid p*Draf-STATmut-lacZ* was constructed by fusing *D-raf* promoter fragment having the base-substituted STAT binding site with the *lacZ* gene in a P-element vector. Transgenic flies bearing the *Draf-STATmut-lacZ* fusion genes were established by P-element mediated transformation. The expression of *lacZ* in transgenic flies bearing *Draf-STATmut-lacZ* fusion genes carrying base substitution in STAT site throughout various developmental stages was extensively reduced in comparison with that in transgenic flies bearing wild type *Draf-lacZ* fusion gene. These results show that the STAT binding site plays an important role in regulation of the *D-raf* gene.

Key words : STAT site Transgenic *Drosophila raf* proto-oncogene Promoter

Introduction

The *raf* proto-oncogene was first identified in the murine sarcoma virus 3611 (MSV-3611) [23]. The activated human *raf* gene was detected from primary stomach cancer [30] and glioblastoma line [11]. Mammalian cells contain 3 *raf* genes encoding Raf-1, A-raf and B-raf [5]. Raf-1, protein product of the c-*raf*-1 gene, is a 70-75 kDa phosphoprotein with a kinase domain occupying the carboxy-terminal half of the molecule and with a potential regulatory domain making up the amino-terminal half and is located primarily in the cytosol [19, 23]. The Raf-1 serves as central intermediate in many signaling pathways, which ultimately regulates cell proliferation, differentiation and development [6, 8] by connecting upstream tyrosine kinase with downstream serine/threonine kinase such as mitogen-activated protein kinase (MAPK) and MAPK kinase (MAPKK) [6, 24]. *Drosophila* carry a single *raf* gene (*D-raf*) in its genome [20]. *D-raf*, a homolog of the human c-*raf-1*, is required in early larval development in proliferation of both somatic and germ cells [1, 20]. Through the *D-raf* mutant phenotype it was found that *D-raf* functions for both the regulation of cell proliferation as mammalian Raf-1 and the determination of cell [1, 13, 21].

In *Drosophila*, promoter regions of the DNA polymerase α and PCNA (proliferating cell nuclear antigen) genes contain a common 8-base pair palindromic sequence TATCGATA, named the DNA replication-related element (DRE) [14, 15, 34]. In previous study, two DRE-like sequence were found in the 5′-flanking region of the *D-raf* gene and it was confirmed that *D-raf* gene is

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regulated by the DNA replication-related element (DRE)/ DRE-binding factor (DREF) system [27].

Members of the JAK (Janus kinase) family of non-receptor tyrosine kinase mediate the cellular response to a large number of cytokines and growth factors in mammalian cells [29]. JAKs associate with the intracellular domains of a large number of cytokine and growth factor receptors. After the extracellular ligand binds to its receptor, the JAK becomes rapidly phosphorylated on tyrosine. They then phosphorylate the receptor, which provides docking sites for latent cytoplasmic transcription factors termed STAT (signal transducer and activator of transcription) proteins [7]. The STATs are activated and then regulate the transcription of immediate-response genes[7].

Four distinct JAKs and six STAT proteins have been identified in mammals [29]. In *Drosophila,* a single JAK homolog, encoded by the gene *hopscotch (hop),* has been identified and shown to be required for normal embryonic patterning and cell proliferation [4]. The *Drosophila* Stat (D-STAT) encoded by the gene *marelle* which is also known as *D-stat* or *stat92E* [37] is a protein of 761 amino acids that resembles members of the STAT family of proteins [37]. D-STAT protein appeared to play an important role in the segmental pattern formation by activating specific stripes of pair rule gene expression and in fly hematopoiesis [16, 37]. STATs recognize related elements consisting of the consensus sequence TTCNNNGAA [16, 37].

In previous study, STAT binding sequence was found in the 5′-flanking region of the *D-raf* gene, and it was demonstrated *in vitro* that *D-raf* gene promoter activity is regulated positively by STAT binding site [18]. In this study, role of the STAT binding site in *D-raf* gene promoter activity was examined *in vivo* using transgenic flies.

Materials and Methods

Oligonucleotide

For obtaining the *D-raf* gene promoter containing the base-substituted STAT binding site, the following oligonucleotide was chemically synthesized (Bio-Synthesis. Inc.)

Draf-STATmut

5′-CGAAATGTAGTAAAATgCGCGcAAAGTAAATAAATTGTTA-3′

Substituted nucleotides are shown by small letters and the potential STAT binding sequence is underlined.

Plasmid construction

To construct the plasmid p5′-663*Draf-STATmut-lacZ*, *D-raf* gene promoter fragment containing the base-substituted STAT binding site was isolated by digesting plasmid p5′-663*Draf-STATmut*-CAT [18] with *Kpn*I. Then the fragment was inserted into *Kpn*I site of the plasmid pCaSpeR-AUG-βgal containing P-element.

Drosophila strains

The *white*(*w*) was employed as a host in P-element transformation method [32]. *w/w;Pm/SM1;Pre/TM3* was used as balancer for p[*w*+;*Draf-STAT*mut] insertion site in the chromosomes of transformants [24]. The *w/w;Draf-lacZ/Draf-lacZ;*+/+ transgenic strain was used as positive control [28].

Establishment of transgenic flies

Germ line transformation was carried out essentially as described by Karess [17]. Embryos of *w* strain were collected, dechorionated, aligned, desiccated, and covered with paraffin oil on a coverslip. The plasmid p5′-663 *Draf-STATmut-lacZ* and helper plasmid pπ25.7wc were dissolved in injection buffer (5 mM KCl, 0.1 mM sodium phosphate, pH 6.8) at final concentration of 0.5 $\mu g/\mu\ell$ and combined in the 5:1 ratio. The p5′-663 *Draf-STATmut-lacZ* and helper plasmid were co-injected into the posterior end of precellular embryo before the pole cells have formed [26]. Each adult derived from an injected embryo (the so-called G_0 generation) should be individually mated with flies of the host strain. The phenotype of the next generation(G_1) was facilitated by the *w*+ marker with variable yellow to red eye color on pCaSpeR-AUG-βgal [35]. The *w;Pm/SM1;Pre/TM3* strain

for balancer chromosomes was used.

X-Gal staining of larvae

The third instar larvae were washed Ringer's solution (182 mM KCl, 46 mM NaCl, 3 mM $CaCl_2$, and 10 mM Tris-HCl (pH 7.2) and were dissected with microforceps. After dissection, tissues were incubated in fixation buffer (26% glutaraldehyde in PBS) for 15 min at room temperature [2]. For photography, tissues were immersed in glycerol, mounted on slides, and photographed with an Olympus micriscope (BX-40).

Quantitative measurement of β-galactosidase activity

Quantitative assay of β-galactosidase activity was carried out with modified method of Simon and Lis [31]. Groups of 20 to 100 individuals each of dechorinated embryos, larvae, pupae, and adult flies were homogenized in 200 $\mu\ell$ of ice-cold assay buffer (50 mM potassium phosphate, pH 7.5/1 mM $MgCl_2$). Each tube was added with an extra 500 $\mu\ell$ of assay buffer and mixed at maximum speed for 30 sec. Homogenates were centrifuged at 10,000 rpm at 4℃ for 10 min. For each assay, 100 to 400 μg of the supernatant was added to give 1 mℓ of assay buffer containing 1 mM chlorophenol red-β-D-galactopyranoside (CPRG) substrate. Reaction incubations were carried out at 37℃ in the dark [2]. Substrate conversion was measured at 574 nm using a spectrophotometer at 0.5, 1, 2 and 3 h after addition of the extract, and the rate of color development was linear. The β-galactosidase activity was defined as absorbance units per mg of protein per hour. To correct for endogenous β-galactosidase activity, extracts from the strain *w* were included in each experiment and endogenous reading was subtracted from readings obtained with each transformant line.

Results and Discussion

D-raf, *Drosophila* homolog of human c-*raf*-1, palys an important role in regulation of cell proliferation and differentiation during development [1, 13, 20]. Multiple regulatory elements should participate in the expression of *D-raf*. In previous study, it was revealed that *D-raf* gene is regulated by DNA replication-related element (DRE)/ DRE-binding factor (DREF) system [27], and that various regulatory elements including E2F-like site and STAT binding site are existed in the *D-raf* promoter region [18].

STAT binding sequence was found in the 5'-flanking region of *D-raf* gene by sequence analysis [18]. The sequence is located at positions -82 to -74 with respect to transcription initiation signal site of the *D-raf* gene (Fig. 1). In previous study, it was demonstrated that *D-raf* gene promoter activity is regulated positively by the STAT binding site [18]. This observation have to be confirmed in living organism and transgenic *Drosophila* provides an appropriate system to characterize transcriptional regulatory elements *in vivo*. In this study, role of the STAT binding site in the *D-raf* promoter activity was examined *in vivo*. For obtaining transgenic flies, the reporter plasmid p*Draf-STATmut-lacZ* was constructed by fusing *D-raf* promoter fragment having the base-substituted STAT binding site with the *lacZ* gene in a P-element vector and then the reporter plasmid was injected into embryos of *Drosophila* strain *w* together

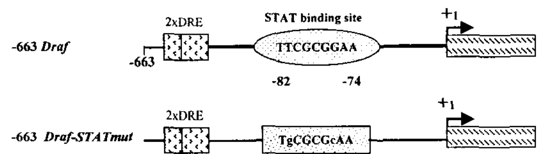

Fig. 1. Structure of 5'-flanking region of the *D-raf* gene and base-substitution mutant in STAT binding site. The lines with horizontal arrows indicated transcriptional initiation site and numbered +1. Locations of each site relative to the transcriptional initiation site are indicated by various numbers. Circle indicate the STAT binding site and overlapped square indicate DRE sequence. Nucleotides substituted for the STAT binding site are shown by small letters.

with helper plasmid Pπ25.7wc. Selection of transgenic flies bearing the *Draf-STATmut-lacZ* fusion gene was facilitated by the *w+* on the vector pCaSpeR-AUG-βgal. Four independent transformant lines were established, and their chromosomal linkages were determined (data not shown). β-Galactosidase activities in adult males of transformant lines were examined by CPRG assay as mentioned in Materials and Methods. All transgenic lines bearing the *Draf-STATmut-lacZ* gene showed lower expression of the *lacZ* than transgenic male bearing wild type *Draf-lacZ* gene [28] (Fig. 2).

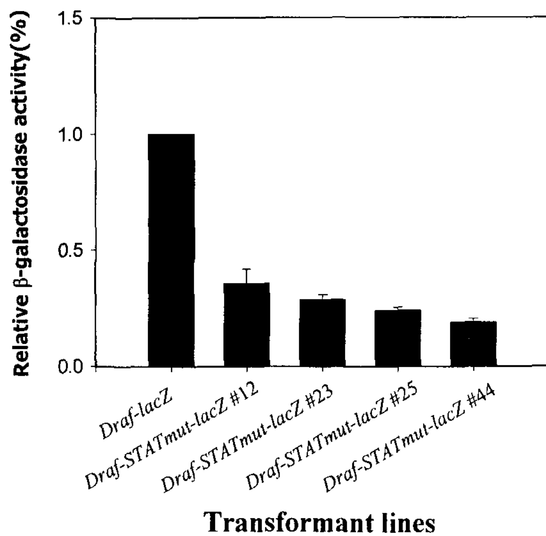

Fig. 2. Expression of the *Draf-STATmut-lacZ* in transformant lines. β-Galactosidase activities in whole-body homogenates of adult males of transgenic lines bearing *Draf-STATmut-lacZ* gene were measured. For each assay, 200 to 400 μg of the supernatant was added to give 1 mℓ of assay buffer containing 1 mM chlorophenol red-β-D-galactopyranoside substrate (CPRG). Reaction incubations were carried out at 37℃ in the dark. Substrate conversion was measured at 574 nm using a spectrophotometer 0.5, 1, 2 and 3 h after addition of extract, and the rate of color development was linear. β-Galactosidase activities in the extracts were expressed as absorbance units/h/mg of protein. Level of expressions were shown as mean±standard error of six different experiments.

To examine role of the STAT binding site in the *D-raf* promoter activity during *Drosophila* development, activities of the mutated promoter monitored by quantitative β-galactosidase assay at various developmental stages were compared with those of the wild type *Draf-lacZ* transgenic flies. As shown in Fig. 3, mutation in the STAT site resulted in extensive reduction of *lacZ* expression throughout all stages of development. Expression level of the *Draf-STATmut-lacZ* fusion gene in larval tissues was compared with that of the wild type *Draf-lacZ* fusion gene by X-gal staining. Transgenic larvae having mutations in STAT binding site had a reduced staining signal in the brain lobe and ganglion, the salivary gland imaginal ring and the fat body (Fig. 4).

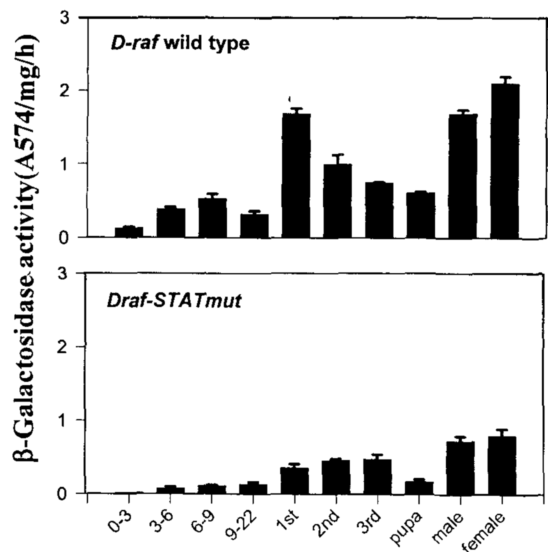

Fig. 3. Effect of base substituted mutations in STAT binding site on *D-raf* promoter activity during development. Male transgenic flies bearing *Draf-lacZ* fusion gene or *Draf-STATmut-lacZ* fusion gene were crossed with female wild type flies and extract were prepared from *Drosophila* bodies at various stages of development. β-Galactosidase activities in the extracts were expressed as absorbance units/h/mg of protein. Level of expressions were shown as mean±standard error of six different experiments.

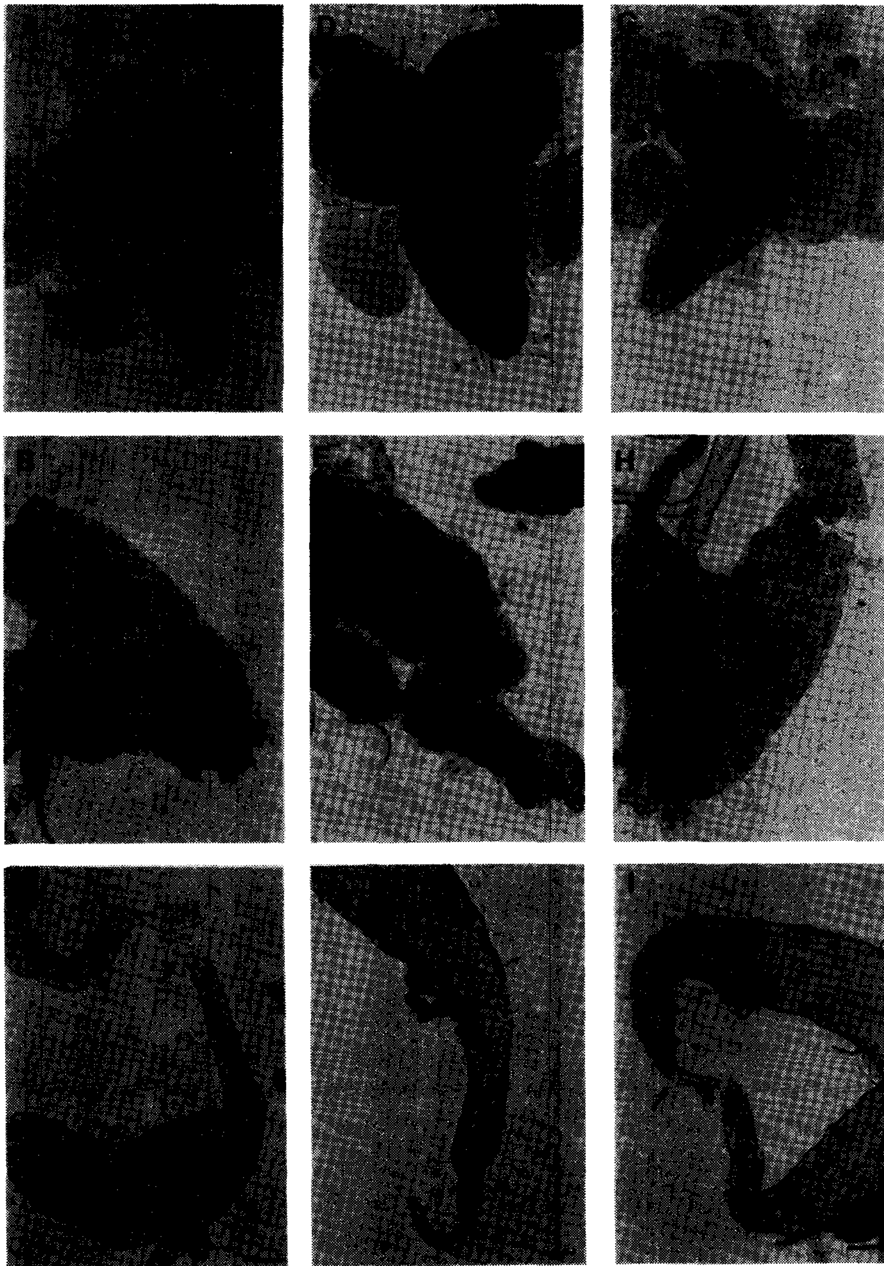

Fig. 4. Demonstration of β-galactosidase activity in the brain lobes and ganglion, the fat bodies and the salivary gland imaginal rings of *Draf-STATmut-lacZ* transgenic third instar larvae. Brain lobes (panels A, D and G), fat bodies (panels B, E and H) and salivary gland imaginal rings (panels C, F and I) were dissected from the third instar larvae. Tissues from *w* strain larvae carrying no transgene were processed in the same way as controls (panels A, B and C). p5'-663*Draf-lacZ* transformants (panels D, E and G) ; p5'-663*Draf-STATmut-lacZ* transfomants (panels G,H and I). Arrow indicated salivary gland imaginal ring. Scales bars=300 ㎛.

In our result, the expression of *lac*Z in transgenic flies bearing the *Draf-STATmut-lacZ* fusion genes carrying base substitutions in the STAT site was extensively reduced in comparison with that in transgenic flies bearing wild type *Draf-lacZ* fusion genes (Fig. 2 and 3). The promoter activity of the *Draf-STATmut* showed 55% *in vitro* [18] and 40% *in vivo,* as compared with that of wild type *D-raf* .

We reported previously the possibility that *D-raf* gene is involved directly or indirectly in immune response processes by demonstrating transcriptional activation of the *D-raf* gene by injury and the abnormality in hemocyte population of hypomorphic mutant $D\text{-}raf^{C110}$ [12], although of which mechanism remains unknown. Transcriptional activation of the *D-raf* gene after injury was detected specically in fat body. *Drosophila* fat body, functional analog tissue of mammalia liver, is the humoral immune system [25] and an important tissue of *Drosophila* immune response. In *Draf-STATmut-lacZ* transgenic larvae, the transcriptional activation by injury was not detected (data not shown). This fact suggests that the STAT binding site of *D-raf* promoter region may have a critical role in transcriptional activation of *D-raf* gene by injury.

Our results demonstrate *in vivo* that the STAT binding site in the 5′-flanking region of the *D-raf* gene plays an important role in regulation of the *D-raf* gene.

Acknowledgements

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초록 : 형질전환 초파리를 이용한 *Drosophila raf* 유전자 발현조절영역에 존재하는 STAT결합부위의 역할에 관한 연구

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STAT들은 다양한 cytokine이나 성장호르몬 등에 의해 활성화되어 핵으로 빠르게 이동되어 유전자 발현을 조절한다. 우리는 *D-raf* 유전자의 5′ 발현조절영역에서 STAT결합부위 염기서열을 발견하였다. 본 연구는 *D-raf* 유전자발현조절에 있어서 STAT결합부위의 역할을 형질전환 초파리를 사용하여 조사하였다. STAT결합부위에 염기치환돌연변이를 도입시킨 *D-raf* promoter부분을 P인자 벡터의 *lacZ*유전자에 융합시킴으로써 리포터 플라스미드 *pDraf-STATmut-lacZ*를 제작하였다. 이 리포터 플라스미드를 이용하여 얻은 *Draf-STATmut-lacZ*형질전환 초파리의 *lacZ*발현을 발생단계별, 조직별로 조사한 결과, 거의 모든 발생단계에서 정상형 *Draf-lacZ* 형질전환 초파리의 *lacZ*발현에 비해 크게 감소하였다. 본 연구결과들은 STAT결합배열 부위가 *D-raf* 유전자발현조절에 있어서 중요한 역할을 함을 개체수준에서 증명하고 있다.