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The *Tol2*-mediated Gal4-UAS method for gene and enhancer trapping in zebrafish

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ABSTRACT

The Gal4-UAS system provides powerful tools to analyze the function of genes and cells *in vivo* and has been extensively employed in *Drosophila*. The usefulness of this approach relies on the *P* element-mediated Gal4 enhancer trapping, which can efficiently generate transgenic fly lines expressing Gal4 in specific cells. Similar approaches, however, had not been developed in vertebrate systems due to the lack of an efficient transgenesis method. We have been developing transposon techniques by using the madaka fish *Tol2* element. Taking advantage of its ability to generate genome-wide insertions, we developed the Gal4 gene trap and enhancer trap methods in zebrafish that enabled us to create various transgenic fish expressing Gal4 in specific cells. The Gal4-expressing cells can be visualized and manipulated *in vivo* by crossing the transgenic Gal4 lines with transgenic lines carrying various reporter and effector genes downstream of UAS (upstream activating sequence). Thus, the Gal4 gene trap and enhancer trap methods together with UAS lines now make detailed analyses of genes and cells in zebrafish feasible. Here, we describe the protocols to perform Gal4 gene trap and enhancer trap screens in zebrafish and their application to the studies of vertebrate neural circuits.

1. Introduction

The yeast transcriptional activator Gal4 has a modular structure consisting of the DNA-binding domain and the transcriptional activation domain [1,2]. Gal4 binds to its specific recognition sequence UAS (for upstream activating sequence) and activates transcription of target genes [3]. Since Gal4 expressed in particular tissues stimulates expression of a gene linked to UAS in a tissue-specific manner, the Gal4-UAS system has been employed to analyze gene functions *in vivo* in *Drosophila*. Namely, the *P* element-mediated enhancer trapping efficiently creates a number of fly lines expressing Gal4 in specific cells, and genes of interest are expressed in a spatially and temporally regulated fashion in the Gal4-expressing cells [4].

The zebrafish is a useful model for genetic studies of vertebrate systems. Several hundreds of fertilized eggs can be obtained from a single mating and a large number of adult fish can be maintained in a limited laboratory space. Due to these advantages, a variety of genetic approaches for investigating gene function have been carried out; i.e., chemical mutagenesis [5,6], retroviral insertional mutagenesis [7–9], target-selected mutagenesis [10,11], zinc-finger nuclease-based mutagenesis [12,13] and morpholino knock-down [14]. In addition to these, targeted gene expression in specific tissues by using the Gal4-UAS system was described in zebrafish [15]. However, the usefulness of the Gal4-UAS method had been limited since construction of transgenic lines expressing Gal4 in various tissues and cells had been laborious and time-consuming, mainly because of the lack of an efficient transgenesis method.

We have been developing transposon techniques by using the *Tol2* transposable element [16]. We cloned a cDNA encoding the transposase protein from the medaka fish *Tol2* element and developed a two-component transposition system [17,18]. When a plasmid carrying the *Tol2* element is injected to zebrafish embryos with the *Tol2* transposase mRNA synthesized *in vitro*, the *Tol2* element is excised from the plasmid and integrated into the genome by the activity of the transposase [19]. Because of the high transposition efficiency in the germ line and the capacity to carry a large DNA fragment [19,20], the *Tol2*-mediated transgenesis has become a popular method to create transgenic zebrafish and has been applied to gene trap and enhancer trap approaches [21–23].

Recently, the *Tol2* transposon system was successfully applied to the Gal4 gene trap and enhancer trap methods in zebrafish [24–27]. First, we constructed a novel Gal4 variant Gal4FF, which we think suitable for transcription activation in zebrafish. Second, we developed gene trap and enhancer trap constructs by using the *Tol2* transposon vector and Gal4FF. Third, we constructed transgenic fish carrying fluorescent reporter genes downstream of UAS and performed screens to identify transgenic fish expressing Gal4 in specific tissues and cells. Finally, we constructed transgenic fish carrying an effector gene, in this case a gene for tetanus toxin, downstream of

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UAS, and demonstrated that our system can inhibit neural functions. Here we describe the protocols to perform the Gal4 gene trap and enhancer trap screens in zebrafish and to apply the Gal4 transgenic fish to the studies of vertebrate neural functions.

2. Methods and materials

2.1. A novel transcriptional activator Gal4FF

We developed a novel transcriptional activator Gal4FF that contains the 147 amino acids DNA-binding domain from Gal4 and two tandem repeats of a 13 amino acids transcription activation module (PADALDDFDLDML) from VP16 (Fig. 1) [28,29]. In previous studies [15,24,25,30–32], the full-length Gal4 or Gal4-VP16 that contained the DNA binding domain and the VP16 activation domain was used for the Gal4-UAS system in zebrafish. We decided to employ Gal4FF since it is less toxic than Gal4-VP16 (see below).

2.2. Gal4FF enhancer trap and gene trap constructs

T2KhspGFF is the enhancer trap construct that contains 638-bp DNA of the *hsp70* promoter region, the Gal4FF gene and the SV40 polyA signal between essential *cis*-sequences of *Tol2* (Fig. 2A and B). T2KSAGFF is the gene trap construct that contains a splice acceptor from the rabbit ß-globin gene, the Gal4FF gene and the SV40 polyA signal between essential *cis*-sequences of *Tol2* (Fig. 2B) [26].

2.3. The UAS plasmid

To facilitate development of UAS reporter and effector fish, we constructed T2MUASMCS that contained five tandem repeats of the Gal4-recognition sequence (5×UAS), a TATA sequence, a multi-cloning site (MCS) and SV40 ployA between essential *cis*-sequences of *Tol2* (Fig. 2C). Any genes cloned at the MCS are expected to be expressed in the presence of Gal4 [26].

2.4. The UAS:GFP reporter line

T2KUASGFP is a reporter construct that contains the EGFP gene downstream of 5×UAS between essential *cis*-sequences of *Tol2* (Fig. 2C). The plasmid carrying the T2KUASGFP construct was injected into fertilized eggs with the *Tol2* transposase mRNA. Eight injected fish were crossed with wild-type fish and the resulting F1 embryos were injected with a Gal4FF expression plasmid DNA. The offspring from five injected fish showed GFP expression. These GFP-positive fish were raised, analyzed by Southern blot analysis, and fish carrying single T2KUASGFP insertions were identified. One of such lines showed ubiquitous GFP expression when crossed with a line expressing Gal4FF ubiquitously and was established as the UAS:GFP reporter line. Inverse PCR analysis revealed that the T2KUASGFP insertion was located within the Nedd4-binging protein 1 gene (NM_199787) [26].

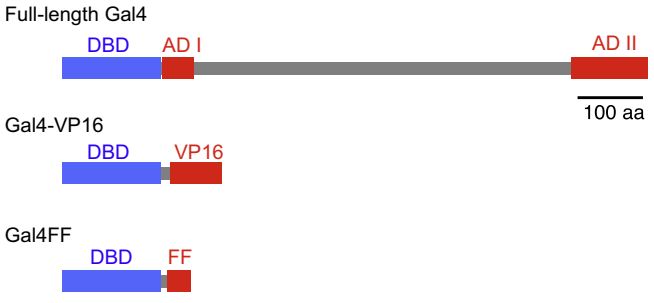

Fig. 1. The structures of full-length Gal4, Gal4-VP16 and Gal4FF. The structures of the full-length Gal4 (top), Gal4-VP16 (middle) and Gal4FF (bottom) are shown. The DNA-binding domain of Gal4 (DBD, 1–147 a.a.) is shown in blue. Transcription activation domains of Gal4 (AD I: 148–196 a.a. and AD II: 768–881 a.a.), a transcriptional activation domain from VP16 (VP16) and two transcription activation modules (FF) are shown in red. The bar corresponds to 100 a.a.

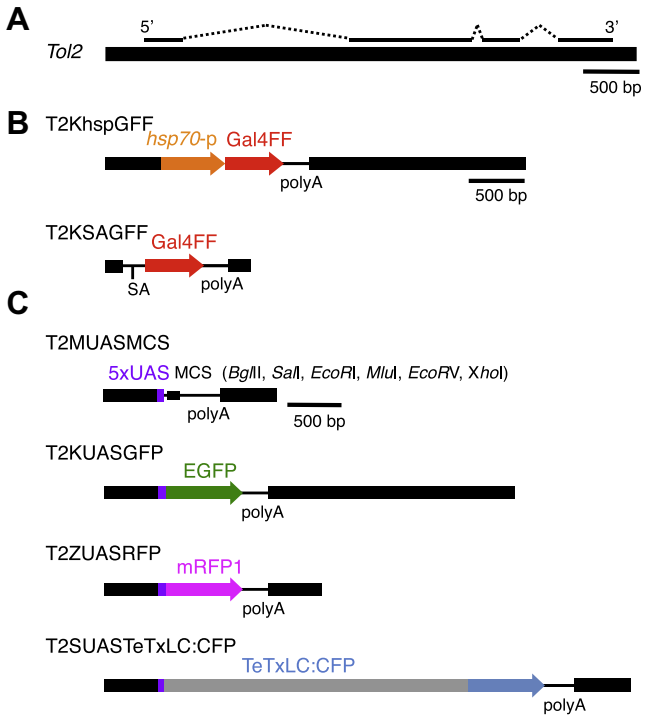

Fig. 2. The *Tol2* transposable element and constructs. (A) The structure of the full-length *Tol2* transposable element. The *Tol2* sequence is shown as a thick black line. Thin lines and dotted lines indicate exons and introns of a gene for the transposase. (B) The structures of the enhancer trap construct T2KhspGFF and the gene trap construct T2KSAGFF. *Tol2* sequences are shown in thick black lines. Arrows indicate the coding sequence for Gal4FF (red) and the zebrafish *hsp70* promoter (orange). SA indicates a splice acceptor from the rabbit ß-globin gene. (C) The structures of UAS constructs. *Tol2* sequences are shown in thick black lines. Arrows indicate the EGFP (green), mRFP1 (magenta) and TeTxLC:CFP fusion (gray and blue) genes. Scale bars indicate 500 bp.

2.5. The UAS:RFP reporter line

T2ZUASRFP was constructed by cloning the mRFP1 gene [33] at the *Xho*I site of the T2MUASMCS plasmid (Fig. 2C). The plasmid carrying T2ZUASRFP was injected to fertilized eggs with the *Tol2* transposase mRNA. Nine injected fish were crossed with the enhancer trap line hspGGFF15, which expressed Gal4FF in the central nervous system, and the resulting F1 embryos were analyzed for RFP fluorescence. The F1 embryos from seven injected fish showed RFP expression. These RFP-positive fish were raised, analyzed by Southern blot analysis, and fish lines carrying single T2ZUASRFP insertions were identified. One of such lines that showed the strongest RFP expression when crossed with the hspGGFF15 fish was established as the UAS:RFP reporter line. Inverse PCR analysis revealed that the T2ZUASRFP insertion was located in a solute carrier protein gene (Slc12a8) [26].

2.6. The UAS-tetanus toxin light chain effector lines

Tetanus toxin light chain (TeTxLC) cleaves a vesicle membrane protein Synaptobrevin and thereby blocks neurotransmitter release from synaptic vesicles [34]. T2SUASTeTxLCCFP was constructed by cloning a gene encoding the CFP-tagged TeTxLC [35]