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Transposon piggyBac mediated Pax6 Expression in Malaria Vector *Anopheles stephensi*

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말라리아 매개 모기 *Anopheles stephensi*에서 트랜스포존 piggyBac을 이용한 Pax6 발현

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ABSTRACT : Pax6, a member of the highly conserved homeobox gene family, is known to be expressed in spatially and temporally restricted pattern during embryogenesis. To examine the spatial expression pattern of Pax6 in malaria vector mosquito *Anopheles stephensi*, in different molecular environment, the germ line transformation technique using piggyBac transposon combined with the use of Pax6 specific 3xP3-EGFP marker was utilized. Four transgenic lines with a transformation rate of 6.7% were established. Transgenes were stably expressed in subsequent several generations. The transgenic lines showed 3 different expression pattern with spatial specificity, possibly due to enhancing and/or silencing position effects. In two transgenic lines, noble expression pattern of Pax6 was observed in the region that has not been previously reported in any animal species. The results show that the tranposon piggyBac mediated germ line transformation system can be used as an efficient tool for the generation of diverse spatially restricted reporter gene expression.

Key words : Pax6, *Anopheles stephensi*, piggyBac, Germ line transformation, 3xP3-EGFP, Enhancing and/or silencing.

요 약 : Pax6는 진화적으로 잘 보존된 homeobox 유전자 그룹의 하나로 배 발생기 동안 시공간적으로 제한되어 발현된다. 이 실험은 말라리아 매개모기인 *Anopheles stephensi*에서의 Pax6 발현을 서로 다른 분자환경 조건에서 조사해 보기 위해 트랜스포존의 하나인 piggyBac과 Pax6에 결합하는 3xP3-EGFP를 사용한 생식세포 형질전환 방법을 사용하였다. 4개의 형질전환 계열이 만들어졌고 형질전환율은 6.7%였으며, 도입 유전자는 여러 세대에 걸쳐 안정적으로 발현되었다. 4계열은 3가지의 공간적 발현 형태를 보였으며 이는 트랜스포존 삽입 위치에 따른 enhancing 혹은 silencing의 결과로 예상된다. 이 결과를 통해 트랜스포존 piggyBac을 사용한 형질전환 시스템은 일반적인 보고자 유전자 발현 실험에서 다양한 형태의 공간적 발현 결과를 유도하는 매우 효율적인 방법으로 사용될 수 있으리라 예상된다.

INTRODUCTION

Pax6 is a highly conserved transcription factor and its gene acts as a master control gene for eye development throughout the animal kingdom. Its expression is limited to the eye, central nervous system, lens placode, nasal epithelium, and pancreas. The vertebrate Pax6 genes code for a subfamily of Pax proteins which show extraordinarily high conservation not only at the sequence but also in their expression pattern(Krauss et al., 1991; Martin et al., 1992; Walther & Gruss, 1991). Consistent with this expression pattern, mutations in the Pax6 gene show a complete failure or abnormalities of such organs in mouse(Hill et al., 1991), *Drosophila melanogaster*(Quiring et al., 1994), and human(Martha et al., 1995).

The expression pattern for Pax6 has been characterized by northern blotting and *in situ* hybridization analysis(Ton et al., 1992). By creating diverse molecular environment, transposable elements provide effective methods for correlating genetic and molecular information. Using several transposons such as her-

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mes, mariner, minos, and piggyBac, the technology to produce transgenic insects has been an effective tools to find and analyze genes, specially for enhancer detection or gene trapping(O'Brochta et al., 2003). piggyBac transposable element was shown to be one of the best candidates as a germ line transformation vector. It is characterized by a high specificity for its target sequence and precise manner of transposon integration, which was well documented in several insect species(Fraser et al., 1995; Handler & Harrell, 2001). Several protocols, including plasmid microinjection(Stuart et al., 1988), have been developed to improve transgenesis frequency and transgene expression.

Berghammer et al.(1999) presented a specific and universal fluorescent transformation marker that is based on the enhanced green fluorescent protein(EGFP; Tsien, 1998), and an artificial promoter(P3) that is responsive to the Pax6. The 3xP3 EGFP Pax gene marker in combination with the transposable element, has been successfully used in several insects, demonstrating its potential to serve as a reliable arthropod marker(Horn et al., 2000; Thomas et al., 2002). Artificially derived Pax6 homodimer binding site P3(Wilson et al., 1995; Czerny & Busslinger, 1995) tells Pax6 gene expression showing EGFP in cells of a wide variety of arthropod organism.

Since little is known about the mechanism that controls expression of the Pax6 genes, it is important to understand the expression pattern of Pax6 in the certain genomic condition provided by animal transgenesis using transposon derived vector. Examining transcriptional regulation of Pax6 will reveal how it functions in different tissues during embryogenesis. Here, I report the expression of the Pax6 in the transformed malaria vector, *Anopheles stephensi* lines using piggyBac transposable element coupled with the strong Pax6 specific 3xP3-EGFP[pBac (3xP3-EGFP)] selectable marker and the possible use of this transgenesis system in the spatial expression pattern study.

MATERIALS AND METHODS

Malaria vector mosquito *Anopheles stephensi* were maintained at 27°C with 12h cycles of light and dark and fed on 10% sucrose. Female mosquitoes were fed blood on Swiss Webster mice. Larvae were reared at 27°C and fed ground fish food. pBac(3x3-EGFP) transformation vector containing the synthetic promoter 3x3, in front of a TATA box regulating the expression of the enhanced green fluorescent protein gene(EGFP), was flanked by the essential terminal sequences of the piggyBac transposable element. The transformation vector(300µg/ml) was co-injected with the piggyBac transposase-encoding helper plasmid phsp-pBac(Handler & Harrell, 1999)(150µg/ml) into preblastoderm *A. stephensi* embryos essentially as described previously(Grossman et al., 2001). Eggs were collected from blood-fed females 72~120h after a blood meal over a period of approximately 30 minutes. Eggs were permitted to age approximately 30 minutes until they were pale gray. Aged eggs were collected, aligned and fixed to a cover slide using a strip of double-sided tape. The eggs were desiccated slightly and covered with Series 27 Halocarbon oil. The oil was removed immediately after injection and cover slip with the injected eggs immersed in a beaker containing deionized water and incubated at 27°C until hatching. Hatched larvae were pooled and reared in conventional mosquito larvae-rearing trays. Emerged adults were sorted by sex and used to establish founder families. Each founder family consisted of around 20 adults originating from the injected embryos(G0) and mated with 60~100 wild type mosquitoes of the opposite sex. Progeny of these families(G1) were screened as young larvae for the presence of tissue expressing the green fluorescence protein. Transposable Element(TE) display, a finger printing method, was performed essentially as described to monitor transposable element movement(Guimond et al., 2003). Single mosquito genomic DNA preparations were prepared using protocol of Ashburner(1989). Genomic DNA from individual adult was isolated and digested with MspI. Adapters consisting of a duplex of oligonucleotides MspIa(5'GA CGATGAGTCCTGAG) and MspIb(5'CGCTCAGGACTCAT) were ligated and PCR reactions were performed. The initial preselective PCR reaction was conducted with the primers MspIa and the piggyBac-specific primers piggyL1(5' TATGAGTTAAATCT TAAAACTCACG) for analysis of the left end and piggyR1 (5'GTGAATTTATTATTATTAGTATGTAAGTG) for analysis of the right end. Preselective reactions were performed in 2.5mM $MgCl_2$ and followed by a round of selective PCR primers MspIa and the Cy5-labelled primers piggyL2Cy5(5'Cy5-CAGTGACAC TTACCGCATTGACAAGC) for analysis of the left end and piggyR2Cy5(5'Cy5-ATATACAGACCGATAAAAACACATGCG) for analysis of the right end. Selective PCR reactions were performed in 2.5mM $MgCl_2$. Reaction products were fractionated on

an 8% polyacrylamide DNA denaturing gel, blotted onto 3MM paper, dried and scanned on a Storm 860 optical scaner (Molecular Dynamics). Reaction band products of interest were excised from the gel, re-amplified using the selective PCR protocol, cleaned with Wizard PCR preps(Promega), and sequenced.

RESULTS

The expression of Pax6 was observed in tissues of *A. stephensi* transgenic larvae carrying a fusion construct of [EGFP/3xP3] piggyBac vectors with EGFP under the regulatory control of the 3xP3 promotor. Transgenic mosquitoes were created by co-injecting pBac(3xP3 EGFP) and phsp-pBac, a plasmid expressing piggyBac transposase, into preblastoderm embryos of the wild-type strain. Of the 793 embryos injected, 117 hatched resulting in 54 adults(G0, male 26 and female 28). G0 adults were used to yield a total of 3076 G1 larvae. Five families were produced transgenic progeny for an estimated transformation frequency of 6.7 %. In order to verify the piggyBac transposition, the insertion of four transgenic lines was analyzed by sequencing flanking genomic regions. DNA sequences of the piggyBac integration sites, from TE display analysis on EGFP positive animals, showed that a characteristic cut-and paste transposition reactions of piggyBac elements into TTAA target sites that were duplicated during integration. Flanking genomic DNA sequences(Table 1) were used in a BLAST search and showed no link to any of the chromosome of *Anopheles gambiae*. As expected the transgenic larvae became markedly bright in their fluorescence due to the expression of the reporter construct. The level of expression did not change during the larval development. Expression of EGFP in transgenic larvae showed 3 different patterns. G1 offspring of the majority(201/205, line 1 and 2) exhibited previously described EGFP expression according to the activity of the 3xP3 promoter. As expected with the 3xP3 promoter control, individuals of these lines(line 1 and 2) had EGFP marker gene expression confined to the eye, brain, ventral nerve cord, and anal papillae(Fig. 1). However, 2% (4/205) of the transgenics featured possible 'enhancer trap' lines which is spatially and/or temporally restricted transgene expression due to regulation imposed by sequences near the insertion site. One line with EGFP expression in the whole body(line 3, Fig. 2a) and one line specifically expressing EGFP band in the pylorus(line 4, Fig. 2b) were identified. The expression of the all transgene is stable in subsequent generations.

Table 1. Integaration site analysis by TE display of transgenic lines showing the first 16 nucleotides flanking the inverted terminal repeats(arrow heads) of the piggyBac vector.

	Left Flank	piggy Bac	Right Flank
Plasmid	CGCAAATCTTTTTTAA	↔	TTAAATAATAGTTTCT
Line 1	AGCGCGAACTTATTAA	↔	TTAAACACGAATTCCA
Line 2	TGTTACGCTTACTTAA	↔	TTAAGAGACTTTTTGA
Line 3		→	TTAAGGATCTTGCTAC
Line 4		→	TTAAAGAAATCGGCGG

Plasmid refers pBac(EGFP-3XP3). Line 1-4 refer transgenic lines.

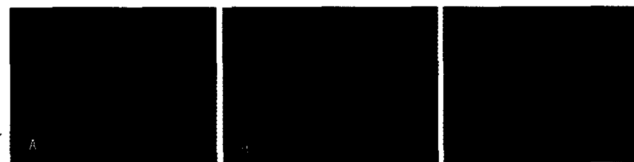

Fig. 1. Wild type of *A. stephensi* larva in third instar and same stage of stable transgenic line 1 and 2 with restricted EGFP expression pattern driven by 3xP3 promoter. Results of these two lines correlate with the previous reports. A. Wild type; B. Dorsal view showing EGFP expression in brain; C. Ventral view in brain, nerve cord, and anal papillae.

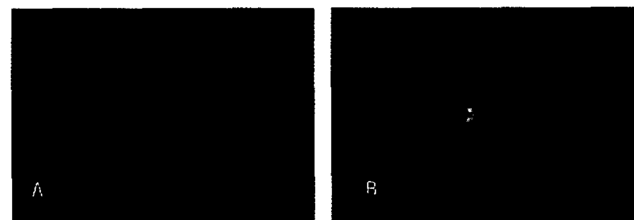

Fig. 2. Fluorescence photomicrographs of specific transgenics, possibly 'enhancer trapped lines', showing noble expression of EGFP. A. Dorsal view of transgenic line 3 larva in third instar. Whole body shows EGFP positive. B. Dorsal view of line 4 larva in third instar. Pyloric area exhibits EGFP expression.

DISCUSSION

In order to investigate the spatiotemporal Pax6 expression

pattern in *A. stephensi,* I made use of the transgenic mosquito lines with EGFP marker gene driven by the three tandem repeats of the P3 site(3xP3 promoter) in the transposon constructs (Berghammer et al., 1999; Horn et al., 2000; Horn & Wimmer, 2000). Results revealed a random pattern of Pax6 gene expression depending on the position of transposon integration site and the influence of transcriptional regulating element.

During embryogenesis, the evolutionary conserved transcriptional regulator Pax6 is expressed, as tissue specific regulatory gene, in the neural tube(Krauss et al., 1991; Martin et al., 1992; Walther & Gruss, 1991) and additionally seen in the developing eye, nasal primordia(Walther & Gruss, 1991) and pancreas (Mansouri et al., 1999). Pax6 proteins recognize their target genes via the DNA-binding function of the paired domain (Kozmik et al., 1992). The paired domain is a highly conserved motif without any obvious sequence homology with other known protein domains. The artificially derived three Pax6 dimeric binding sites 3xP3 promoter used in present study was designed to bind Pax6 homodimers(Sheng et al., 1997). After multimerization, P3 mediates tissue specific gene expression where Pax6 transcription factor behaves as a regulator.

In this study, all individuals of produced transgenic lines strongly expressed EGFP in specific tissues and was continuously observed during larval, pupal, and adult stages. In two lines, 3xP3 promoter drived EGFP expression in the eye, brain, ventral nerve cord, and anal papillae. It is consistent with the well documented expression of Pax6 in several insects(Horn et al., 2000; Hediger et al., 2001). These results with 3xP3-EGFP marker confirm the presence of Pax6 activity in those tissues. However, in two other transgenic lines, noble expression of the transgene was observed. Significant expression was detected in tissues of pylorus, in line 3, which is not known to express Pax6. Moreover, in transgenic line 4, expression was observed in the whole body of the animal as well as in the previously reported area.

It was reported that same fluorescence pattern were detected, in eyes, nervous system, and anal plates, in transgenic fly lines generated with constructs based on four transposons: P-element (Rubin et al., 1982), mariner(Medhora et al., 1988), Hermes (Warren et al., 1994), and piggyBac(Cary et al., 1989). This seems to show that all depicted pattern do not result from enhancer traps or expression mediated by transposon backbones. However, in present lines, certain aspects of the previous fluorescence pattern in mosquitoes are suppressed or ex-suppressed, which is most likely due to developmental stage or tissue specific position effects. There are various regulatory elements i.e. transcription factors and/or enhancers that regulate temporal and spatial gene expression, and these can be mixed and matched. In many cases, the genes for transcription factors are activated by other transcription factors. Since all 4 transgenic lines showed EGFP expression in specific tissues, it was expected that the 3xP3 promoter would mediate fluorescence in these tissues. Thus, it is possible that there are additional factors required to allow the 3xP3 promoter to work. The fluorescence in line 3 and 4 could be driven by different transcriptional activators, as line 1 and 2 do not express there. It is also possible that 3xP3 might bind to other paired class type homeodomain transcription factors, which is responsible for the fluorescence in the pylorus or whole body, as well as Pax6.

The tissue specific and temporal expression of Pax6 gene may be controlled by a combination of different *cis*-regulating elements upstream and downstream of the initiation site. There have been accumulated reports of the role of *cis* regulatory elements in specific gene expression. Enhancers are the major determinant of differential transcription in space and time. A gene can have several enhancer elements, each turning it on in a different set of cells. If the gene becomes expressed, that means that it has come within the domain of an active enhancer. It is reported that some distinct *cis* elements are actually involved in regulating the two promoters of Pax6 and in tissue specific expression of Pax6 in the eye, the central nervous system, and pancreas(Prosser & van Heyningen, 1998). Another region upstream of the promoter was known to contain a regulatory element that directs Pax6 expression in the developing photoreceptor(Schmahl et al., 1993). Several experiments also indicated that some signals produced by specific tissue can repress the region specific expression of Pax6 in the developing tissue(Goulding et al., 1993). The cell specific silencer in combination with other positive and negative elements may contribute to tissue-specific and temporal expression of the Pax gene. It means that cis regulatory elements are responsible for selective activation of the Pax6 promoter in cells that can express Pax6 mRNA. Enhancer trapping can occur randomly during development and place a new gene next to a particular regula-

tory element. According to Kammandel et al.(1999), there are two major promoters for Pax6 gene and four enhancer regions, in mouse, that activate the Pax6 gene in the pancreas, lens, neural tube, and retina, respectively. In enhancer trapping, a transposon encoded reporter gene expressed from a promoter is regulated by genomic sequences flanking the insertion site (Bellen et al., 1989; Bier et al., 1989). This suggests that addition of a transposon containing a functional coding region to an endogenous locus that encodes a nonfunctional protein might complement the mutant phenotype. In line 3 and 4 of present experiment, the expression pattern of the reporter could mimic that of a nearby gene, because the transposon has come under the control of the same regulatory influences as the endogenous gene. This might be the classical enhancer traps showing enhanced EGFP expression at a restricted area controlled by 3xP3 promoter. In other transgenic lines, the reporter gene expression appeared to be specifically silenced in all but the enhanced tissues. Thus, application of promoter allows detection of both enhancing and silencing properties of regulatory elements. Similar to the P-element of *Drosophila*(Tsubota et al., 1985), piggyBac integration might be favored in the vicinity of transcriptional regulatory elements. These regions are frequently comprised of control elements that maintain independent realms of gene activity(Vigdal et al., 2002). Some of these might also cohabit with transcriptional enhancers or silencers(Boulikas, 1995).

Present results showed that the enhancement of transgenesis frequencies combined with the random generation of novel expression pattern using the piggyBac transposon system allows the fast and simple generation of a wide range of diverse EGFP expression pattern. Thus, a set of transgenic lines expressing EGFP in diverse structures or organs can be established and used for further molecular work without a major effort to the characterization of promoter elements. It will offer the chances to create various experimental conditions for understanding the molecular mechanism that control spatiotemporal expression of the Pax6 genes.

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