ABSTRACT

Regulated expression systems have been extremely useful in developmental studies, allowing the expression of specific proteins in defined spatial and temporal patterns. If these proteins are fused to an appropriate molecular tag, then they can be purified or visualized without the need to raise specific antibodies. If the tag is inherently fluorescent, then the proteins can even be visualized directly, in living tissue. We have constructed a series of P element-based transformation vectors for the most widely used expression system in Drosophila, GAL4/UAS. These vectors provide a series of useful tags for antibody detection, protein purification, and/or direct visualization, together with a convenient multiple cloning site into which the cDNA of interest can be inserted.

INTRODUCTION

The ability to detect, localize, and purify proteins is central to cell biology and biochemistry. The increasing pace of molecular studies, particularly the identification of many novel proteins by the genome sequencing projects, demands an efficient, reliable method to supplement the traditional approach of raising and purifying specific antibodies. If the tag is inherently fluorescent, allowing the tagged protein to be purified by Ni affinity chromatography (IMAC) (10,15). The final tag, EGFP, a mutant variant (F64L and S65T) (5) of green fluorescent protein (GFP) from Aequoria victoria, allows direct visualization of the tagged protein in living or fixed cells by fluorescence microscopy (7).

To maximize the utility of these vectors for developmental studies, we based them on the pP[UAST] GAL4/UAS expression vector. The bipartite GAL4/UAS expression system is a powerful tool for studying the effect of ectopic expression in Drosophila (2). In this system, the cDNA of interest is subcloned into a vector such as pP[UAST] (Figure 1A). This places the cDNA under the control of a minimal Hsp70 promoter, with multiple binding sites for the Saccharomyces cerevisiae transcription factor GAL4. The pP[UAST] vector also provides a polyadenylation sequence from simian virus 40 (SV40) and a mini-white gene and cis-acting P element sequences for Drosophila transformation by P element-mediated germ-line transformation. Wild-type Drosophila do not have a GAL4-like activity, so the cDNA is not expressed unless GAL4 is provided. It is therefore possible to make stable transformed lines carrying toxic cDNAs, such as activators or inhibitors of key signaling pathways. Separately, fly lines expressing GAL4 in defined developmental patterns can be established. Many such lines are now available. When the GAL4 and UAS-cDNA lines are crossed, the F1 progeny express the cDNA in the defined pattern of the GAL4 line.

MATERIALS AND METHODS

Plasmid Construction

The sequences of the oligonucleotides used as linkers and for PCR are shown in Table 1. We eliminated the single Ndel site of pP[UAST] by digestion, end-filling, and re-ligation to be able to use Ndel in our multiple cloning sites. We then took two complementary oligonucleotides (UAS-ATG+ and UAS-ATG−; see Table 1), allowed them to anneal, and ligated them to EcoRI-NotI digested pP[UAST] (from which the Ndel site had been removed) to make pP[UAS-LP] (Figure 1B). These manipulations introduce a consensus translation start sequence (4,12) and a downstream Ndel site, with the ATG of the Ndel site in frame with the start codon.

We amplified inserts from pGEMHA, pGEMMH, and pGEM-T-EGFP (6) using Pfu DNA polymerase (Stratagene Europe, Amsterdam, The Netherlands) according to the manufacturer’s instructions and the oligonucleotides listed in Table 1. Each gel-purified PCR product was incubated with T4 DNA polymerase (Roche Diagnostics Ltd., Lewes, East Sussex, UK) in the presence of dGTP and dCTP (Amerham Pharmacia Biotech UK Ltd., Little Chalfont, Bucks, UK) but not dATP or dTTP. This created an Ndel-compatible cohesive end at one end of the fragment and an EcoRI-compatible cohesive end at the other end. These fragments were
then subcloned into NdeI-EcoRI-digested pP[UAS-LP] to create pP[UAS-HA], pP[UAS-HM], and pP[UAS-EGFP], respectively (Figure 1C). The final structure of each molecular tag vector is shown in Figure 1.

**Immunological Methods**

For immunoblotting, protein extracts from flies were separated by SDS-PAGE and transferred to PVDF membrane (Immobilon™ P; Millipore, Bedford, MA, USA) by standard methods. HA was detected with a mouse monoclonal α-HA antibody (clone 12CA5) or actin with a mouse monoclonal α-actin antibody (clone C4; ICN Biomedicals, Aurora, OH, USA) at a final concentration of 1:500 and 1:1,000,000, respectively. Horseradish peroxidase-coupled secondary antibody (Sigma, St. Louis, MO, USA) was used at a final concentration of 1:7500. All antibodies were used in PBS supplemented with 0.1% Tween® 20 (v/v) and 5% fat-free dried skimmed milk (w/v). The secondary antibody was visualized using enhanced chemiluminescence SuperSignal® kit (Pierce Chemical, Rockford, IL, USA).

Embryos were collected over a period of 16 h. They were fixed and stained as described in Reference 9. Embryos were mounted in 85% glycerol containing 2.5% n-propyl gallate and examined using a Bio-Rad Radiance Plus confocal scanning microscope (Bio-Rad Laboratories, Hercules, CA, USA). Tubulin was detected with a rat monoclonal α-tubulin antibody (clone YL1/2; Sero-tec Ltd., Kidlington, Oxford, UK) at a final concentration of 1:200. Endogenous Nipp1Dm was detected using a rabbit polyclonal α-Nipp1Dm antibody at a concentration of 1:50. FITC- and Cy5-conjugated secondary antibodies were obtained from Jackson Immunoresearch Laboratories (West Grove, PA, USA). All antibodies were used in PBS supplemented with 0.1% Tween 20 (v/v) and 5% fetal calf serum (v/v).

**RESULTS AND DISCUSSION**

The GAL4/UAS vector pP[UAST] provides transcriptional but not translational control sequences. We therefore modified pP[UAST] to provide a consensus translation start sequence with a convenient multiple cloning site. NdeI is particularly convenient for subcloning complete coding regions as it contains ATG in its recognition sequence (CATA/TG). We used pP[UAS-LP] as the basis for three molecular tag vectors. We wanted two different epitope tags, recognized by different monoclonal antibodies, to facilitate co-localization and co-immunoprecipitation studies. We also wanted an oligo-histidine tag to allow purification by IMAC and an enhanced

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**Figure 1. Vectors for the expression of tagged proteins in Drosophila.** (A) The structure of pP[UAST] and derivatives. The NdeI site of pP[UAST], which we eliminated, is marked. Other functional elements are as pUAST (2,3). The cDNA of interest is inserted into the multiple cloning site (MCS), shown in detail in panel B. (B) The sequence of the multiple cloning sites of pP[UAST] and its derivatives. All sites marked are unique apart from NcoI. pP[UAS-tag] represents all three vectors encoding molecular tags. Sequences in bold highlight the changes made in the construction of each vector relative to its precursor. (C) The sequence of the molecular tags. The start codon and NdeI site (CATATG, in bold) correspond to the start codon and NdeI sites of pP[UAS-tag] in panel B. NcoI sites in the HA and HM tags are underlined. All sequences shown have been confirmed by sequencing.
GFP (EGFP) tag to allow direct visualization of the tagged protein. We obtained suitable tags from available yeast expression vectors (6). pP[UAS-HA] encodes three copies of the HA epitope, pP[UAS-HM] encodes 6×His and two copies of the myc epitope, and pP[UAS-EGFP] encodes EGFP (Figure 1).

These vectors are intended to enable the controlled expression of tagged protein. It is important that the expression level is very low in the absence of GAL4, that the function of the protein is not disrupted by the tag, and that the tag performs as expected. We tested these properties by expressing the protein phosphatase type 1 catalytic subunit PP1β9C (8,13) using pP[UAS-HA] and Nipp1Dm, a Drosophila homologue of the mammalian PP1 inhibitor NIPPI (unpublished data and References 1 and 16), in pP[UAS-HA] and pP[UAS-EGFP].

Using standard breeding techniques, we constructed a fly strain carrying arm-GAL4 (arm-Gal4) and UAS-HA-PP1β9C on the third chromosome, balanced over TM6B. arm-GAL4 produces low-level, ubiquitous expression of GAL4 (14). These flies were crossed to y cho sn flw/Y;arm-GAL4, UAS-HA-PP1β9C/+ (UAS-HA-PP1β9C) to complement the PP1β9C hemizygotes carried the rescue construct rather than TM6B. Thus, HA-PP1β9C can perform all essential functions of wild-type PP1β9C (8,13). Transgenic flies carrying UAS-HA-PP1β9C were constructed by standard techniques. UAS-HA-PP1β9C and arm-GAL4 insertions on the third chromosome were recombined together. The recombined arm-GAL4, UAS-HA-PP1β9C constructs result in low-level constitutive expression of HA tagged PP1β9C (Figure 2). Male flies carrying this chromosome (wY; arm-GAL4, UAS-HA-PP1β9C/TM6B) were crossed to heterozygous flw females (y cho sn flw/FM7c, B). All emerging flw hemizygotes carried the rescue construct rather than TM6B. Thus, HA-PP1β9C can perform all essential functions of wild-type PP1β9C. TM6B interacts with FM7c in males to reduce the number recovered in this class. Female flies are equally viable with or without arm-GAL4, UAS-HA-PP1β9C, showing that expression of HA-PP1β9C at this level does not significantly affect viability.

Table 2. UAS-HA-PP1β9C Is Functional

<table>
<thead>
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<th>Genotype</th>
<th>Total No. Flies</th>
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<tr>
<td>y cho sn flw/Y;arm-GAL4, UAS-HA-PP1β9C/+</td>
<td>41</td>
</tr>
<tr>
<td>y cho sn flw/Y;TM6B/+</td>
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<tr>
<td>FM7c, B/Y; arm-GAL4, UAS-HA-PP1β9C/+</td>
<td>34</td>
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<tr>
<td>FM7c, B/Y;TM6B/+</td>
<td>14</td>
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<tr>
<td>y cho sn flw/w;rm-GAL4, UAS-HA-PP1β9C/+</td>
<td>58</td>
</tr>
<tr>
<td>y cho sn flw/w;TM6B/+</td>
<td>57</td>
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<tr>
<td>FM7c, B/w;arm-GAL4, UAS-HA-PP1β9C/+</td>
<td>42</td>
</tr>
<tr>
<td>FM7c, B/w;TM6B/+</td>
<td>46</td>
</tr>
</tbody>
</table>

We tested the ability of a HA-tagged PP1β9C cDNA expression construct (UAS-HA-PP1β9C) to complement the PP1β9C mutant flw (13). Transgenic flies expressing UAS-HA-PP1β9C were constructed by standard techniques. UAS-HA-PP1β9C and arm-GAL4 insertions on the third chromosome were recombined together. The recombined arm-GAL4, UAS-HA-PP1β9C constructs result in low-level constitutive expression of HA tagged PP1β9C (Figure 2). Male flies carrying this chromosome (wY; arm-GAL4, UAS-HA-PP1β9C/TM6B) were crossed to heterozygous flw females (y cho sn flw/FM7c, B). All emerging flw hemizygotes carried the rescue construct rather than TM6B. Thus, HA-PP1β9C can perform all essential functions of wild-type PP1β9C. TM6B interacts with FM7c in males to reduce the number recovered in this class. Female flies are equally viable with or without arm-GAL4, UAS-HA-PP1β9C, showing that expression of HA-PP1β9C at this level does not significantly affect viability.

Figure 2. Detection of HA tag in protein extracts. Western blots of protein (0.5 fly per lane) from wild-type (lane 1), UAS-HA-PP1β9C only (lane 2), arm-GAL4, UAS-HA-PP1β9C (lane 3), and arm-GAL4 only (lane 4) were probed with antibodies against HA to detect HA-PP1β9C or against actin as a loading control. An immunoreactive protein of the expected size of approximately 39 kDa was detected in extracts from arm-GAL4, UAS-HA-PP1β9C flies but not from wild-type, UAS-HA-PP1β9C or arm-GAL4 flies.
EGFP-Nipp1Dm in live embryos, thus avoiding possible fixation artifacts and allowing the study of changes in subcellular localization of the tagged protein in individual cells in real time rather than by comparison of fixed specimens.

We have subsequently used these vectors to express a wide range of proteins in *Drosophila* and found that in every case the molecular tags perform as expected. Therefore we believe that these convenient vectors will be of general utility to the *Drosophila* community.

REFERENCES

Reverse Transcription Slippage over the mRNA Secondary Structure of the LIP1 Gene

BioTechniques 31:1286-1294 (December 2001)

INTRODUCTION

The synthesis of cDNA from mRNA has become a common practice in the amplification of nucleic acid sequences by PCR and the construction of a cDNA library. During the synthesis of the first-strand cDNA, the presence of stable secondary structures in mRNA often cause early termination of the reaction. Thus, it is difficult to reach the cap site (5′ end) of these mRNA species (6). In an attempt to determine the 5′-untranslated region (UTR) of a novel gene, we found that, during the first-strand cDNA synthesis, the reverse transcriptases jumped over the RNA secondary structures and continued the cDNA synthesis, resulting in shortened forms of cDNA.

The novel cellular protein was identified through yeast two-hybrid screening using the latent nuclear antigen (LNA) encoded by ORF73 of Kaposi’s sarcoma-associated herpesvirus (KSHV) as bait. The novel cellular protein, LNA-interacting protein 1 (LIP1), is a 418-amino acid polypeptide encoded by an ORF of 1257 nucleotides (5). No homologous gene with known function was found in our nucleotide database search. Northern-blot analysis showed that LIP1 transcripts were present in all tissues tested (5). In an attempt to map the full-length LIP1 transcripts, we performed RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) (3) to identify the sequence of 5′-UTR of the transcripts. In RLM-RACE, the RNA template is treated with alkaline phosphatase to remove free 5′-phosphates from RNA or contaminating DNA molecules. Tobacco acid pyrophosphatase (TAP) is then added to remove the cap structure from mRNA. Target gene-primed reverse transcription, followed by nested PCR amplification, is then carried out to amplify the 5′ end of the full-length specific target transcript. Here, we report that the 5′-UTR of LIP1 forms secondary structures that were skipped over by the reverse transcriptases during the first-strand cDNA synthesis under standard conditions. Optimizations of the reaction conditions with high temperature and a thermostable reverse transcriptase resulted in the successful reverse transcription through the entire 5′-UTR sequences.

MATERIALS AND METHODS

RLM-RACE

To obtain the 5′-UTR sequences of capped LIP1 mRNA, RLM-RACE was performed using the FirstChoice™ RLM-RACE Kit (Ambion, Austin, TX, USA), following the manufacturer’s instructions. Total RNA was isolated.