# Transcriptional and posttranscriptional regulation of the gene for Dally, a *Drosophila* integral membrane proteoglycan

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Received 6 March 2001; accepted 21 March 2001

First published online 30 March 2001

Edited by Jesus Avila

**Abstract** ***division abnormally delayed* (*dally*), a *Drosophila* member of the glypican family, has been implicated in Dpp and Wg signaling. Here, we report the genomic structure and regulation of the *dally* gene. The *dally* gene is composed of nine exons, and its expression is controlled by a TATA-less promoter. Analysis of transgenic flies bearing the *dally* promoter fused to the *lacZ* reporter gene showed that a 371 bp sequence of the *dally* 5′ flanking region was capable of mimicking the patterns of *dally* enhancer trap expression in developing tissues, including embryonic epidermis and imaginal discs. The tissue-specific enhancers that drive marker gene expression in embryo and the wing disc are mapped in the 5′ upstream region of *dally* gene. We propose that *dally* gene expression is also regulated posttranscriptionally by controlling the translation efficiency and stability of its mRNA.** 

*Key words:* Proteoglycan; Gene expression; Transgenic animal; Translational regulation; mRNA stability; *Drosophila melanogaster*

## 1. Introduction

Proteoglycans, which bear glycosaminoglycans attached to specific serine residues of the protein core, are found abundantly on the cell surface and in the extracellular matrix. Among these, heparan sulfate proteoglycans (HSPGs), in particular, have drawn much attention because of their important roles in growth factor signaling, cell adhesion, cell migration, and viral infection [1]. For example, a number of studies using tissue culture systems have established that the cell surface-associated HSPGs are required for several growth factors including fibroblast growth factor and transforming growth factor-β (TGF-β) to mediate signals to the target cells, serving as co-receptors for growth factor signaling [2].

Glypicans are a family of integral membrane HSPGs anchored to the cell membrane via a glycosylphosphatidylinositol linkage [3]. Six members of the glypican family (GPC1–6) have been identified in humans as well as in mice, all of which are expressed in a tissue-specific fashion. A high level of expression of GPC3 was detected in liver, lung, and kidney but not in brain in humans [4]. The expression patterns of glypicans parallel those of several growth factors and their receptors. In the developing murine limb, the expression pattern of GPC3 is similar to that of BMP4, and it regulates cellular responses to BMP4 signals [5]. These studies suggested that the regulated expression of proteoglycan co-receptors is responsible for normal growth factor signaling. Recently, the promoter sequences of rat GPC1 and human GPC3 have been isolated and shown to be controlled by TATA-less promoters [6,7]. Deletion analysis of the GPC1 promoter revealed that an approximately 200 bp fragment immediately upstream of the transcriptional initiation site drives its efficient transcription. However, the molecular mechanism underlying the spatial and temporal regulation of glypican gene expression remains to be elucidated.

*dally* encodes a *Drosophila* member of the glypican family of HSPGs, and is expressed in specific patterns in most imaginal discs as well as embryos [8–10]. A series of genetic experiments demonstrated that *dally* participates in two distinct signaling pathways in a developmental stage- and/or tissue-specific manner. *dally* is required for signaling mediated by Decapentaplegic (Dpp), a *Drosophila* member of the TGF-β superfamily, during development of several imaginal tissues [11]. In embryonic development, however, *dally* is expressed in a segmental pattern and mediates signaling directed by Wingless (Wg), a member of the Wnt family [9,10]. Ectopic expression of the *dally* gene perturbs the signaling of these growth factors, resulting in abnormal morphogenesis [9,11]. These results indicated that precise regulation of *dally* gene expression is essential for a normal cellular response to Dpp and Wg.

To understand the molecular mechanisms that account for the regulation of *dally* gene expression, we characterized the structure and function of the *dally* promoter. Studies using transgenic animals identified regulatory regions that are responsible for *dally* expression in wing disc and embryonic epidermis. In addition, we propose the possibility that *dally* gene expression is also regulated posttranscriptionally by modulating translation efficiency and mRNA stability.

## 2. Materials and methods

### 2.1. Plasmid construction

The fusion gene consisting of the 5′ upstream region of the *dally* gene and the *Escherichia coli lacZ* structural gene (*dally*[3619]-*lacZ*) was constructed as follows: the 4.5 kb *Bam*HI–*Xba*I fragment of the *dally* genomic clone, containing the *dally* 5′ flanking region and the first exon, was subcloned into the *Bam*HI–*Xba*I site of pBluescript. The translation initiation site of *dally* was deleted by exonuclease III, and the resultant fragment containing 111 bp of the *dally* 5′ untrans-

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0014-5793/01/$20.00 
PII: S0014-5793(01)02347-X

lated region (UTR) sequence was subcloned into the pCaSpeR vector, which bears the *lac*Z gene downstream of the linker sequence [12]. A series of deletion constructs, each bearing a different length of the 5′ flanking region, was made from *dally*[3619]-*lac*Z.

To construct *dally*-5′ UTR-*lac*Z, the *dally* 5′ flanking region and 5′ UTR (from −1147 to +560) were amplified by polymerase chain reaction (PCR) with the *dally* gene-specific primer (5′-GCTGGTACCTTCTGCATGCTGTCTCTGG-3′) and the M13 primer using a *dally* genomic clone that was subcloned into pBluescript as a template. The PCR products were subcloned into pCaSpeR. The *dally*-m5′ UTR-*lac*Z containing the mutated 5′ UTR of *dally* was prepared in the same manner, except that we used a mutated primer (5′-GCTGGTACCTTCTGCCTGCTGTCTCTGG-3′), in which the ATG codon had been eliminated by replacement of the adenine residue with cytosine (underlined).

In order to obtain *dally*-*lac*Z-*dally* 3′ UTR, the *dally* 3′ UTR was amplified by PCR and the SV40 3′ UTR of the *dally*[1147]-*lac*Z construct was replaced by the PCR product.

*2.2. Cell culture and assays of reporter gene expression*

S2 cells were grown in Schneider medium (Sigma) with 12.5% fetal calf serum on a 60 mm diameter tissue culture plate. Exponentially growing cells were transfected with appropriated DNA constructs by the method of calcium phosphate coprecipitation [13]. β-Galactosidase activity in the cell extracts was assayed as previously described [14]. To normalize the various transfection efficiencies, the control vector containing luciferase cDNA under the regulation of the *Drosophila* actin promoter (*act-luc*) was cotransfected with *dally-lac*Z constructs. A luciferase assay was carried out using a PicaGene (Toyo Ink Inc.) according to the manufacturer's protocol.

*2.3. Germline transformation and β-galactosidase activity staining*

The *dally-lac*Z constructs were introduced into flies by P-element-mediated transformation with the *y w* stock as recipients. For each construct, two to five independent lines were analyzed. To detect β-galactosidase activity, third instar larval imaginal discs and embryos were fixed with 8% glutaraldehyde in phosphate-buffered saline and subjected to standard X-gal color reaction for 12 h at 37°C.

## 3. Results and discussion

*3.1. Structure of the dally gene and the dally 5′ flanking region*

To analyze the structure of the *dally* gene, a *Drosophila* P1 phage library (GenomeSystems Inc.) was screened using the *dally* cDNA as a probe. Out of several clones we screened, one clone, DS00932, was found to include the whole structure of the *dally* gene and this was subjected to further analyses. Sequencing of this clone together with information from the *Drosophila* database in the Berkeley *Drosophila* Genome Project revealed that the *dally* gene stretches over an approximately 61 kb region on the genome and is divided into nine exons (Fig. 1A).

The transcription initiation site of the *dally* gene was determined to be an adenine residue (+1 in Fig. 1B) by primer extension analysis (data not shown). The sequence surrounding the transcription start site matches the *Drosophila* consensus sequence for an initiator 'TCA(G/T)T(T/C)' (+1 site is underlined) [15]. A typical TATA box was not found in the *dally* 5′ flanking region, suggesting that this gene is controlled by a TATA-less promoter as observed with several mammalian glypican genes. Instead, the sequence GGTTCGT (+28 to +34) is similar to the downstream promoter element (DPE), which is known as a downstream analog of the TATA box [15]. Previous studies have shown that the DPE appears to be common core promoter along with the TATA box in *Drosophila* [16]. Several putative binding sites for homeodomain proteins, Fushi-tarazu (Ftz) and Even-skipped (Eve) occur in the *dally* 5′ upstream region. This is consistent with a previous report that *dally* is regulated by these selector homeoproteins in late embryogenesis [17].

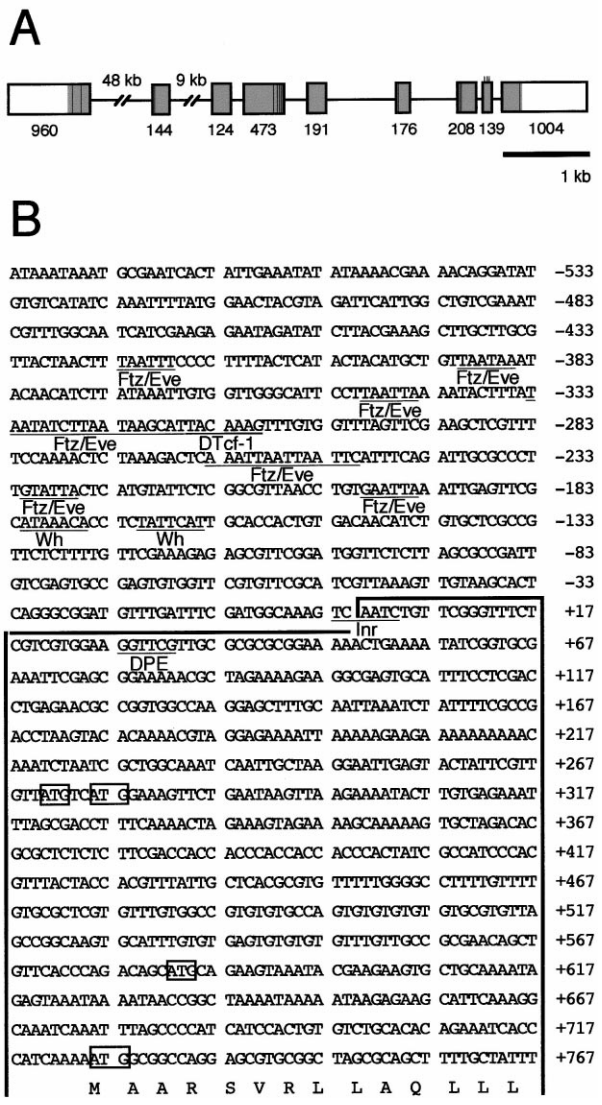


Fig. 1. The exon/intron structure and 5′ flanking region of *dally* gene. A: The exon/intron structure of the *dally* gene. Exons are boxed, and the protein coding regions are shadowed. The lines extending up from the exon represent potential glycosaminoglycan attachment sites. The vertical lines within exons indicate the conserved positions of cysteine residues. The numbers under the exons indicate the length of each exon. B: The sequence around the transcription initiation site of the *dally* gene. The adenine residue at the transcription start site is numbered as +1. The potential transcription factor binding sites are underlined and labeled under the sequence as follows: Ftz/Eve, Fushi-tarazu/Even-skipped; Wh, Winged helix; Inr, initiator; DPE, downstream promoter element. The upstream AUG codons and the initiation codon are boxed. The sequence data have been submitted to DDBJ/EMBL/GenBank under the accession numbers AB052365, AB052366, and AB052367.

*3.2. Tissue-specific enhancers that control dally gene expression*

To analyze tissue-specific enhancer activities in the *dally* 5′ flanking region, spatial and temporal patterns of reporter gene expression in transgenic flies bearing a series of the deletion DNAs of the *dally* 5′ sequence fused to the *lac*Z gene (*dally-lac*Z constructs) were followed. The expression patterns of the *dally*[3619]-*lac*Z transgene in the transformant were indistin-

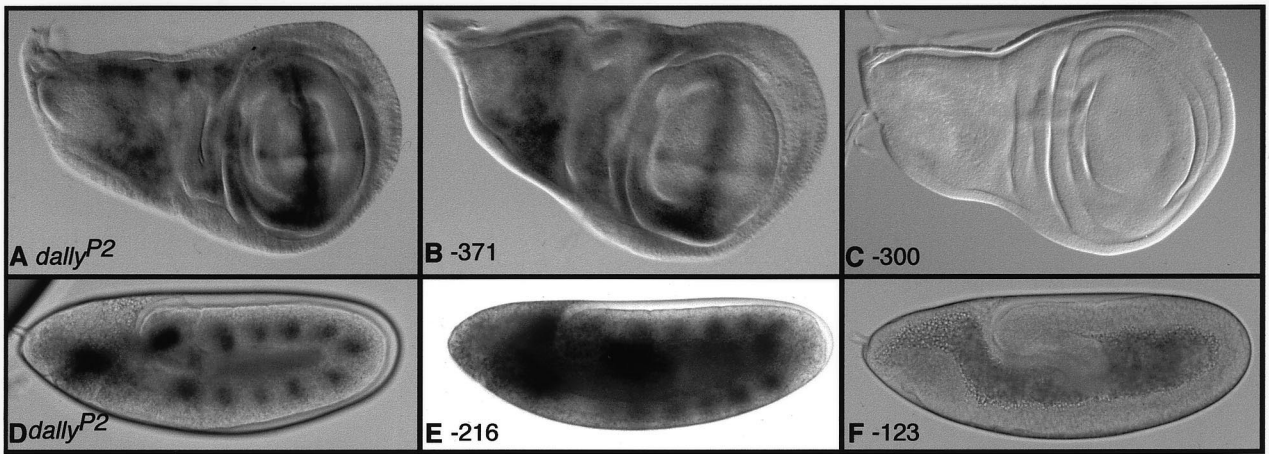


Fig. 2. β-Galactosidase activity staining of *dally*-*lac*Z transgenic animals. The expression patterns of *lac*Z in animals, transformed with *dally*-*lac*Z constructs, were compared with those of the *dally* enhancer trap line. A–C: Third instar larval wing discs are shown for the *dally* enhancer trap line, *dally*$^{P2}$ (A), *dally*[371]-*lac*Z (B), and *dally*[300]-*lac*Z (C). Discs are oriented with their anterior side up and dorsal to the left. D–F: Embryos at stage 9 are shown for *dally*$^{P2}$ (D), *dally*[216]-*lac*Z (E), and *dally*[123]-*lac*Z (F). Anterior is to the left, dorsal up.

guishable from those of the *dally* enhancer trap in many tissues, including embryonic epidermis, imaginal discs, and larval central nervous system (CNS) (data not shown). For example, during embryonic development of the transgenic animals, *dally*[3619]-*lac*Z transgene expression was detected in a segmental pattern as is observed in *dally*$^{P2}$ (Fig. 2). At late third larval instar, the reporter gene was expressed along the dorsoventral (D/V) and anteroposterior (A/P) compartment boundaries of the wing blade.

Removal of the −3619 to −372 region of the *dally* gene did not affect the expression patterns of the marker gene, indicating that the 5′ flanking 371 bp sequence of the *dally* gene is sufficient to sustain the expression patterns of the *dally* enhancer trap in developing wing disc, CNS, and embryonic epidermis (Table 1). Deletion of the region from −371 to −300, however, eliminated *lac*Z expression in wing discs (Fig. 2A–C). In the wing pouch, *dally* enhancer trap expression at the A/P and D/V boundaries is regulated by Wg and Hedgehog (Hh), respectively (Fujise et al., unpublished data). These facts imply that the region from −371 to −300 contains elements which can respond to the signaling mediated by Wg and Hh. The phenomenon is consistent with the observation that this wing enhancer contains the recognition sequence for DTcf-1, a Wg-dependent transcription factor (Fig. 1B). On the other hand, *dally*[300]-*lac*Z and *dally*[216]-*lac*Z transgenes support the embryonic expression (Table 1, Fig. 2D,E). This pattern was lost when the deletion was extended to −123 (Table 1 and Fig. 2F), indicating that the 93 bp sequence between −216 and −123 of the gene is responsible for *dally* expression in embryos. This fragment contains a Ftz/Eve binding site and two sites for transcription factors with the winged helix DNA binding domain such as Sloppy paired 1 and 2 (Slp1/2) [18]. Liang and Biggin [17] proposed that a large fraction of genes including *dally* are directly regulated by Ftz and Eve in embryogenesis. It has also been demonstrated that the *slp* genes are required for maintenance of *wg* expression and repression of *engrailed*, *hh*, *eve*, and *ftz* [19]. It is possible that *dally* expression at embryonic stages is regulated by these transcription factors as has been shown for other segment polarity genes [17,19].

Table 1
Summary of *dally*-*lac*Z expression in transformants

| Transgene | *n* | Expression | | |
|---|---|---|---|---|
| | | Wing disc | CNS | Embryo |
| *dally*[3619]-*lac*Z | 5 | + | + | + |
| *dally*[1147]-*lac*Z | 5 | + | + | + |
| *dally*[445]-*lac*Z | 5 | + | + | + |
| *dally*[371]-*lac*Z | 3 | + | + | + |
| *dally*[300]-*lac*Z | 3 | ND | ND | + |
| *dally*[216]-*lac*Z | 3 | ND | ND | + |
| *dally*[123]-*lac*Z | 5 | ND | ND | ND |
| *dally*[42]-*lac*Z | 2 | ND | ND | ND |
| *dally*[9]-*lac*Z | 2 | ND | ND | ND |

Third instar larval wing discs, larval CNS, and embryos at stage 9 were stained for β-gal activities. Each transgene contains the *dally* upstream sequence with lengths shown in brackets. *n*: number of lines tested. +: reporter gene expression was detected in the same pattern with *dally* enhancer trap expression. ND: reporter gene expression was not detected.

### 3.3. *dally* 5′ UTR downregulates the translation efficiency of *mRNA*

As depicted in Fig. 1B, three AUG codons were found in

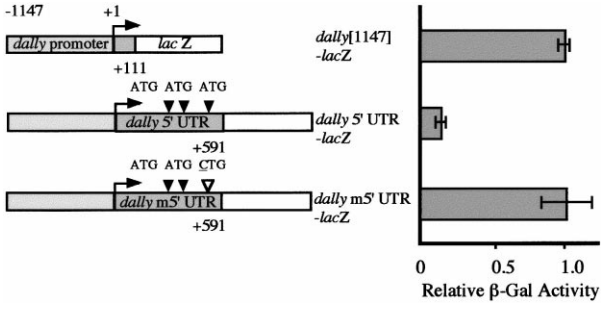


Fig. 3. *dally* mRNA 5′ UTR controls *dally* translation efficiency. The black and white arrowheads represent AUG codons and the mutated AUG codon (CTG) in the *dally* 5′ UTR, respectively. *dally*-*lac*Z constructs were introduced into S2 cells, and β-galactosidase activities of these cells were assayed. The *act*-*luc* construct was cotransfected and used for the normalization of transfection efficiency. Results represent the mean ± S.E.M. for three independent experiments.

A

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CTCTTTAGTA GCTGTAGTTA AGGATGCTGC CCCAGCCCAA GGGATTGAAC  2597
 L  F  S   S  C  S  *

ACAAAAAACG CAATGCGCCC CACTGTGCGA GGGCAATGAA TGATGTAACC  2647
AGAAGAAGAG CAACAGCAGA TGCACACGAA TCGAGTTAAA TCATTAATTA  2697
ATAACTATTA TCATTATTAT TATTATTTGT GTAATTCTTA AGTTGTTACA  2747
AGTAATTTAT AGGCCCTAAG CGCATTCATC TAGCAATCGA CTAACAACTT  2797
TACAAACCAG AAGCGAACCG TGAAACCCTT GAAGTGCACC TCTCCTTCTA  2847
GCTTTTCCAA ATTTGATGCT CATCGAACTT CCATGCCGAA TCCAACTAGA  2897
CTTGTGTGCC ATATAAATGC GTTGGATATT TGCATACGAA TCGAGCAATT  2947
GCTAAACACA ACTAACTATA AACGAAAATT AAACTAAATA GGCTCGCAAT  2997
CAAGCAGAAT CCCTTTTCCA TAGAGTGTGC AGTTTGTTCA GATACATGTT  3047
AATATATATA GCGTACAGTA CACAAAGCTC AATGTTCAAA GCAAGTTGTT  3097
CCTTTTCGGT GTAATTACAC AAGTAAACAG ATCTAAAATC AGAGGCGTGG  3147
CACCACATTC AATTGCTAGG TTTTTTGAGG GCGATCACAA TATATTATAT  3197
ACGTAAAGGA TATAAATCTG TTAGCTGTAG CTCAGTGAAT GTTAAACTAA  3247
AATGAAATTA TTGTTGTCTA CACCTTTCGA ACTGATCGAA TGCGAACAAT  3297
TTTGAAATTG ATCAATTGGG TAATCGAAAT GCGAAATGCA TACGAATGGA  3347
ATTTAGTTGA GTAAACGCAC GGAAAAACTA GAAGACTTAC AGCTTTATAC  3397
GCAAACAAAA AAAAAAAAAA AA                                 3419
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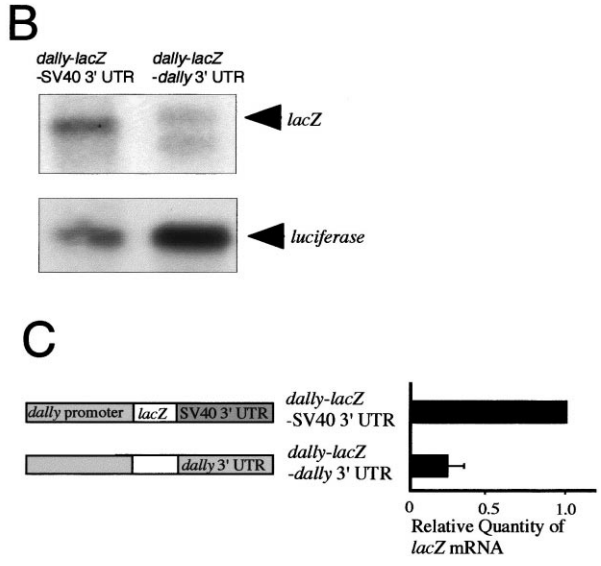


Fig. 4. *dally* 3′ UTR negatively regulates mRNA level. A: The sequence of *dally* 3′ UTR is shown. Numbers at the right side indicate the nucleotides numbered from the transcription start site. An asterisk shows the position of the stop codon. Pentanucleotide sequences (AUUUA), representing a putative mRNA destabilizing motif, are underlined. The Bearded box is double underlined. B,C: Effect of *dally* 3′ UTR on mRNA levels was analyzed by transient transfection in S2 cells. Two *dally-lacZ* constructs, one with control SV40 3′ UTR (*dally-lacZ*-SV40 3′ UTR) and another with *dally* 3′ UTR (*dally-lacZ-dally* 3′ UTR), were transfected in S2 cells. Poly-$(A)^+$ RNAs from transfected cells were analyzed by Northern blotting using either *lacZ* (upper panel) or luciferase (lower panel) as probes (B). Levels of *lacZ* mRNA were quantified by NIH image and normalized by levels of luciferase mRNA. The relative quantities of *lacZ* mRNA for each construct are depicted in the bar graph (C). The value for *dally-lacZ*-SV40 3′ UTR was set at 1.0. Bars represent ± S.E.M.

the *dally* 5′ UTR, each of which has a short open reading frame and is out of frame with Dally. It has been suggested that translation efficiency can be negatively regulated by short upstream open reading frames (uORFs) in 5′ UTR by competing with the actual translation initiation codon [20]. Although only 5–10% of eukaryotic mRNAs contain uORFs, over 65% of mRNA from genes involved in cellular growth and differentiation bear these elements. To examine if *dally* uORFs can affect translation efficiency, we made *dally-lacZ* constructs with or without *dally* 5′ UTR, denoted respectively as *dally*-5′ UTR-*lacZ* and *dally*[1147]-*lacZ*. These constructs were introduced into S2, a *Drosophila* tissue culture cell line, and translation efficiency was analyzed by measuring the *lacZ* mRNA level and relative activities of β-galactosidase. As shown in Fig. 3, insertion of *dally* 5′ UTR caused a significant reduction of β-galactosidase activity. The levels of *lacZ* mRNA as determined by Northern hybridization were not affected (data not shown), indicating that the difference in β-galactosidase activity between two transformants is unrelated to the amount of mRNA.

Out of three uORFs in *dally* 5′ UTR, the third one starting at +547 from the transcription start site contains the sequence homologous to the *Drosophila* translation initiation consensus, (C/A)AA(A/C)AUG [21]. When we mutated this AUG to CTG (*dally*-m5′ UTR-*lacZ*), β-galactosidase activity of the transformant was restored to the level observed in that with the *dally*[1147]-*lacZ* transgene (Fig. 3). These results strongly suggest that one of the uORFs in the *dally* 5′ UTR reduces the translation efficiency of *dally* mRNA.

### *3.4. dally 3′ UTR affects mRNA level*

The 3′ UTR of *dally* mRNA consists of 833 nucleotides of AU-rich sequence and contains two AUUUA motifs, which have been suggested to influence mRNA lability (Fig. 4A) [22]. It also carries the Bearded (Brd) box, which has been shown to affect the steady-state levels of both RNA and protein in *Drosophila* [23]. Identification of these motifs in *dally* 3′ UTR prompted us to determine if *dally* 3′ UTR might affect mRNA accumulation. We made two *dally-lacZ* constructs, one with control SV40 3′ UTR (*dally-lacZ*-SV40 3′ UTR) and another with *dally* 3′ UTR (*dally-lacZ-dally* 3′ UTR). S2 cells were transfected with these two constructs, and the *lacZ* mRNA levels of the transfected cells were analyzed 48 h after transfection. The *act-luc* construct was cotransfected to normalize transfection efficiency. Northern hybridization analysis showed that the level of *lacZ* mRNA from cells transfected with *dally-lacZ-dally* 3′ UTR was significantly lower than that with the control plasmid (Fig. 4B,C). We also obtained a similar result using constructs with *Drosophila* heat shock 70 protein promoter instead of the *dally* promoter (data not shown). These results indicate that *dally* 3′ UTR reduces the *dally* mRNA level in a promoter-independent mechanism, although they do not rule out the possibility that this sequence affects transcription.

Taken together, our results strongly suggest that the expression of the *dally* gene is regulated at multiple steps including transcription, translation, and mRNA degradation. It has been shown that Dpp and Wg signaling pathways are very sensitive to the level of *dally* function and that *dally* can alter the patterning activity of these signaling molecules in a gene dosage-dependent manner [9,11]. These observations imply the intriguing possibility that the precise regulation of the level of Dally protein at the cell surface might contribute to morphogen activity by modulating signaling strength.

*Acknowledgements:* We are grateful to S. Tomino for critically read-

ing the manuscript. This work was supported in part by the Human Frontier Science Program and a Grant-in-Aid for Scientific Research on Priority Area No. 10178102 from the Ministry of Education, Science, Sports, and Culture of Japan. M.T. was supported by Research Fellowships of the Japanese Society for the Promotion of Science for Young Scientists.

## References

[1] Tumova, S., Woods, A. and Couchman, J.R. (2000) Int. J. Biochem. Cell Biol. 32, 269–288.
[2] Schlessinger, J., Lax, I. and Lemmon, M. (1995) Cell 83, 357–360.
[3] David, G. (1993) FASEB J. 7, 1023–1030.
[4] Pilia, G. et al. (1996) Nature Genet. 12, 241–247.
[5] Paine-Saunders, S., Viviano, B.L., Zupicich, J., Skarnes, W.C. and Saunders, S. (2000) Dev. Biol. 225, 179–187.
[6] Asundi, V.K., Keister, B.F. and Carey, D.J. (1998) Gene 206, 255–261.
[7] Huber, R., Schlessinger, D. and Pilia, G. (1998) Gene 214, 35–44.
[8] Nakato, H., Futch, T.A. and Selleck, S.B. (1995) Development 121, 3687–3702.
[9] Tsuda, M. et al. (1999) Nature 400, 276–280.
[10] Lin, X. and Perrimon, N. (1999) Nature 400, 281–284.
[11] Jackson, S.M., Nakato, H., Sugiura, M., Jannuzi, A., Oakes, R., Kaluza, V., Golden, C. and Selleck, S.B. (1997) Development 124, 4113–4120.
[12] Thummel, C.S., Boulet, A.M. and Lipshitz, H.D. (1988) Gene 74, 445–456.
[13] Fehon, R.G., Kooh, P.J., Rebay, I., Regan, C.L., Xu, T., Muskavitch, M.A. and Artavanis-Tsakonas, S. (1990) Cell 61, 523–534.
[14] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1987) John Wiley and Sons, New York.
[15] Burke, T.W. and Kadonaga, J.T. (1997) Genes Dev. 11, 3020–3031.
[16] Kutach, A.K. and Kadonaga, J.T. (2000) Mol. Cell. Biol. 20, 4754–4764.
[17] Liang, Z. and Biggin, M.D. (1998) Development 125, 4471–4482.
[18] Grossniklaus, U., Pearson, R.K. and Gehring, W.J. (1992) Genes Dev. 6, 1030–1051.
[19] Cadigan, K.M., Grossniklaus, U. and Gehring, W.J. (1994) Proc. Natl. Acad. Sci. USA 91, 6324–6328.
[20] Kozak, M. (1991) J. Cell Biol. 115, 887–903.
[21] Cavener, D.R. (1987) Nucleic Acids Res. 15, 1353–1361.
[22] Shaw, G. and Kamen, R. (1986) Cell 46, 659–667.
[23] Lai, E.C. and Posakony, J.W. (1997) Development 124, 4847–4856.