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Overexpressed *Drosophila* DNA Methyltransferase 2 Isoform C Interacts with Hsp70 *in Vivo*

Karim Roder[†,*]

Institute of Molecular Biology, Academia Sinica, Nankang, Taipei 115, Taiwan, Republic of China

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Shen and colleagues (Lin *et al.*, 2004) have recently shown that overexpression of the *Drosophila* DNA methyltransferase 2 isoform C, dDnmt2c, extended life span of fruit flies, probably due to increased expression of small heat shock proteins such as Hsp22 or Hsp26. Here, I demonstrate with immunoprecipitations that overexpressed dDnmt2c interacts with endogenous Hsp70 protein *in vivo* in S2 cells. However, its C-terminal half, dDnmt2c(178-345) forms approximately 10-fold more Hsp70-containing protein complexe than wild-type dDnmt2c. Overexpressed dDnmt2c(178-345) but not the full length dDnmt2c is able to increase endogenous mRNA levels of the small heat shock proteins, Hsp26 and Hsp22. I provide evidence that dDnmt2c(178-345) increases Hsp26 promoter activity *via* two heat shock elements, HSE6 and HSE7. Simultaneously overexpressed Hsp40 or a dominant negative form of heat shock factor abrogates the dDnmt2c(178-345)-dependent increase in Hsp26 transcription. The data support a model in which the activation of heat shock factor normally found as an inactive monomer bound to chaperones is linked to the overexpressed C-terminus of dDnmt2c. Despite the differences observed in flies and S2 cells, these findings provide a possible explanation for the extended lifespan in dDnmt2c-overexpressing flies with increased levels of small heat shock proteins.

Keywords: DNA methyltransferase 2, Heat shock, Heat shock factor, Heat shock protein, Promoter

Introduction

In vertebrates, DNA methylation at cytosines is involved in various biological processes such as chromosome X inactivation, genomic imprinting, cancer development, aging and silencing of retrotransposons (Ponger and Li, 2005). The active vertebrate DNA methyltransferases (Dnmts), Dnmt1, Dnmt2, Dnmt3a and Dnmt3b are a family of proteins with highly conserved motifs in their carboxyl regions (Ponger and Li, 2005). Whereas Dnmt1 is the maintenance enzyme, Dnmt3a and Dnmt3b carry out the *de novo* methylation reaction. Unlike the above three enzymes, Dnmt2 lacks the N-terminal regulatory region (Ponger and Li, 2005). Although Dnmt2 is the most widespread DNA methyltransferase among eukaryotes, only few studies addressed its biological role. Strains of *M. musculus*, *D. melanogaster* and *A. thaliana* with Dnmt2 knockouts were viable, fertile and morphologically indistinguishable from wild-type counterparts (Goll *et al.*, 2006). Several groups have shown that eukaryotic Dnmt2 is responsible for genome methylation (Reddy *et al.*, 2003; Tang *et al.*, 2003; Kunert *et al.*, 2003; Hermann *et al.*, 2003). Very recently, Bestor and colleagues (Goll *et al.*, 2006) demonstrated that Dnmt2 methylated $tRNA^{Asp}$ *in vivo*, a function conserved among species. Furthermore, Shen and colleagues (Lin *et al.*, 2004) provided strong evidence that dDnmt2c is required for the maintenance of the normal life span of fruit flies. Strikingly, overexpression of dDnmt2c extended the life span of flies, probably due to the observed upregulation of small heat shock proteins such as Hsp22 and Hsp26. This finding is supported by a recent report on the overexpression of Dnmt2 (Ehmeth) in the protozoan parasite *Entamoeba histolytica* (Fisher *et al.*, 2006) leading to an increase in Hsp70 transcripts, resistance to oxidative stress and accumulation of multinucleated cells. In the present work, I set out to address the link between dDnmt2c and heat shock proteins in *Drosophila* S2 cells.

[†]Present address: School of Life Sciences, Heriot-Watt University, Edinburgh, EH14 4AS, UK.

*To whom correspondence should be addressed.
Tel: 44-131-451-3464; Fax: 44-131-451-3009
E-mail: k.roder@hw.ac.uk

Materials and Methods

Expression vectors. All constructs were created by using standard molecular biology techniques. Detailed descriptions and plasmid maps will be made available upon request. pAc-Gal and pAc-Gal-dDnmt2c have been described previously (Tang *et al*., 2003). The cDNAs of dDnmt2c encoding amino acids 1 to 177 and amino acids 178 to 345 were amplified by PCR and cloned into pAc-Gal to get pAc-Gal-dDnmt2c(1-177) and pAc-Gal-dDnmt2c(178-345), respectively. pAc-Myc and pAc-Flag contain respective epitope tags downstream of a Kozak sequence with ATG start codon in the multiple cloning site of pAc5.1/V5-HisA (Invitrogen) to allow the expression of 5' epitope-tagged fusion proteins. The plasmids, pAc-Flag-dDnmt2c, pAc-Flag-dDnmt2c(1-177) and pAc-Flag-dDnmt2c (178-345), encode respective Flag-tagged dDnmt2c fusion proteins. Using PCR, the open reading frame of fly Hsp40B1 was cloned in frame with the 5' Flag tag resulting in pAc-Flag-Hsp40B1. The plasmid, pAc-Myc-Hsf(1-537) encodes a myc-tagged, shortened form of *Drosophila* Hsf. The truncation of the last 154 codons removes the C-terminal *trans*-activation domains but leaves DNA-binding and trimerization domains (Wang, Y. *et al*., 2004). The myc-tagged Hsp70Aa fly protein is encoded by pAc-Myc-Hsp70Aa. The plasmid, pCI-Flag contains a Flag epitope tag downstream of a Kozak sequence with ATG start codon in the multiple cloning site of pCI (Promega). The open reading frame of the human Dnmt2 isoform A, hDnmt2a, was cloned into pCI-Flag, resulting in pCI-Flag-hDnmt2a.

Luciferase reporter constructs. The plasmid, pGL3-α was constructed as described previously (Roder *et al*., 2000). pGL3-Hsp26(-671/+167), pGL3-Hsp26(-399/+167), pGL3-Hsp26(-321/+167), pGL3-Hsp26(-179/+167) and pGL3-Hsp26(-42/+167) plasmids contain various sequences of the *Drosophila* Hsp26 gene (relative to the transcription start site, +1) upstream of the luciferase reporter gene of pGL3. Site-directed mutagenesis was used to change the core of the heat shock elements, HSE6 ($^{-346}$5'-GAA-3'$^{-344}$ to 5'-TCC-3') and HSE7 ($^{-367}$5'-GAA-3'$^{-365}$ to 5'-TCC-3') in the construct pGL3-Hsp26(-399/+167) resulting in pGL3-Hsp26(-399/+167)-HSE6m and pGL3-Hsp26(-399/+167)-HSE7m, respectively. The pGL3-α-2xHsp26(-399/-300) plasmid contains two copies of the heat shock-responsive region of Hsp26 linked to the α globin promoter of pGL3-α. Its derivative, pGL3-α-2xHsp26(-399/-300)-HSE6m/HSE7m has mutated HSE6 and HSE7 sites as aforementioned.

RT-PCR. Total RNA was isolated from S2 cells using TRIzol (Invitrogen). The RNA samples were digested with DNAse I and 3 μg RNA reverse transcribed using Superscript II (Invitrogen) and oligo(dT) following the recommendations of the manufacturer. PCR conditions were 95°C for 2 min, then 22 (beta-tubulin) or 25 cycles (dDnmt2c, Gal4-dDnmt2c(178-345), Hsp22, Hsp26) of 95°C for 30 s, 55°C for 30 s, 72°C for 60 s, and finally 5 min at 72°C. Primer sequences will be made available upon request. For semi-quantitative purposes (Alway *et al*., 2001; Lin *et al*., 2004), preliminary experiments were conducted with each PCR product (Hsp22, Hsp26) to ensure that the number of cycles represented a linear portion of the PCR optical density curve for samples from transiently transfected S2 cells. For Hsp22 and Hsp26, the cDNAs from both samples were simultaneously amplified using aliquots from the same PCR mixture. PCR products were separated on 2% agarose gels, stained with ethidium bromide and the corresponding signals were recorded by the IS1000 Digital Imaging System. Next, the band intensities were quantitated using a densitometer. All RT-PCR signals were normalized to the beta-tubulin signal of the corresponding cDNA product to provide a semi-quantitative measure of the relative changes in gene expression. Additionally, 18S primers were used as internal controls giving similar results (data not shown). For statistical analysis, experiments were independently performed three times using RNA samples from independently transfected S2 cells.

Cell culture and transfections. For transient transfections of S2 cells, cells were plated onto 12-well plates at 1.5×10^6 cells/well and transfected by the calcium phosphate method 6 to 18 h later. Each well received 100 ng luciferase reporter construct, 1000 ng expression plasmid and 400 ng CMV-beta-galactosidase. The medium was changed 16 h later and cells were harvested 48 h after transfection. Transfections were performed in triplicates.

(Co-)Immunoprecipitations. In a 10 cm dish, 2.2×10^7 S2 cells were transiently transfected either with 15 μg Flag-tagged expression vector and 10 μg plasmid DNA as carrier (for immunoprecipitations) or with 10 μg Flag-tagged expression vector and 10-20 μg myc-tagged expression vector (for co-immunoprecipitations). The medium was changed 16 h later and cells were harvested 48 h after transfection. Similarly, 293T cells were transiently transfected with 10 μg Flag-tagged expression vector in 10 cm dishes. To crosslink protein, cells were incubated with 1.2 mM Dithiobis(succinimidyl) propionate (DSP, dissolved in DMSO; Sigma) in PBS for 5 min at RT prior to addition of 1 M glycine, pH 7, and cell lysis with NP40 buffer (1% NP40, 150 mM NaCl, 50 mM Tris, pH 8.0). 1-2 mg of protein were incubated with anti-FLAG M2-agarose (Sigma) for 2 h. The supernatant was removed and the beads extensively washed with NP40 buffer prior to adding SDS sample buffer. Western blotting was performed using an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences), with the primary (anti-myc, anti-Flag, anti-Hsp70 from Santa Cruz) and secondary antibodies prepared in Tris-buffered saline solution containing 5% dry milk.

Statistical analysis. Data are expressed as the mean ± SD and statistical comparisons were made using unpaired Student's *t* tests as appropriate.

Results

The C-terminal half of dDnmt2c increases the level of endogenous Hsp22 and Hsp26 mRNAs. Recently, Shen and colleagues (Lin *et al*., 2004) have shown that overexpression of *Drosophila* dDnmt2c extended the life span of flies, probably due to increased expression of small heat shock proteins such as Hsp22 and Hsp26. Overexpression of Hsp26, for example, increased the life span of fruit flies by 30% (Wang *et al*., 2004). To study the mechanism of Hsp26 upregulation by dDnmt2c I planned to use *Drosophila* S2 cells. Transient co-transfections of an expression vector for

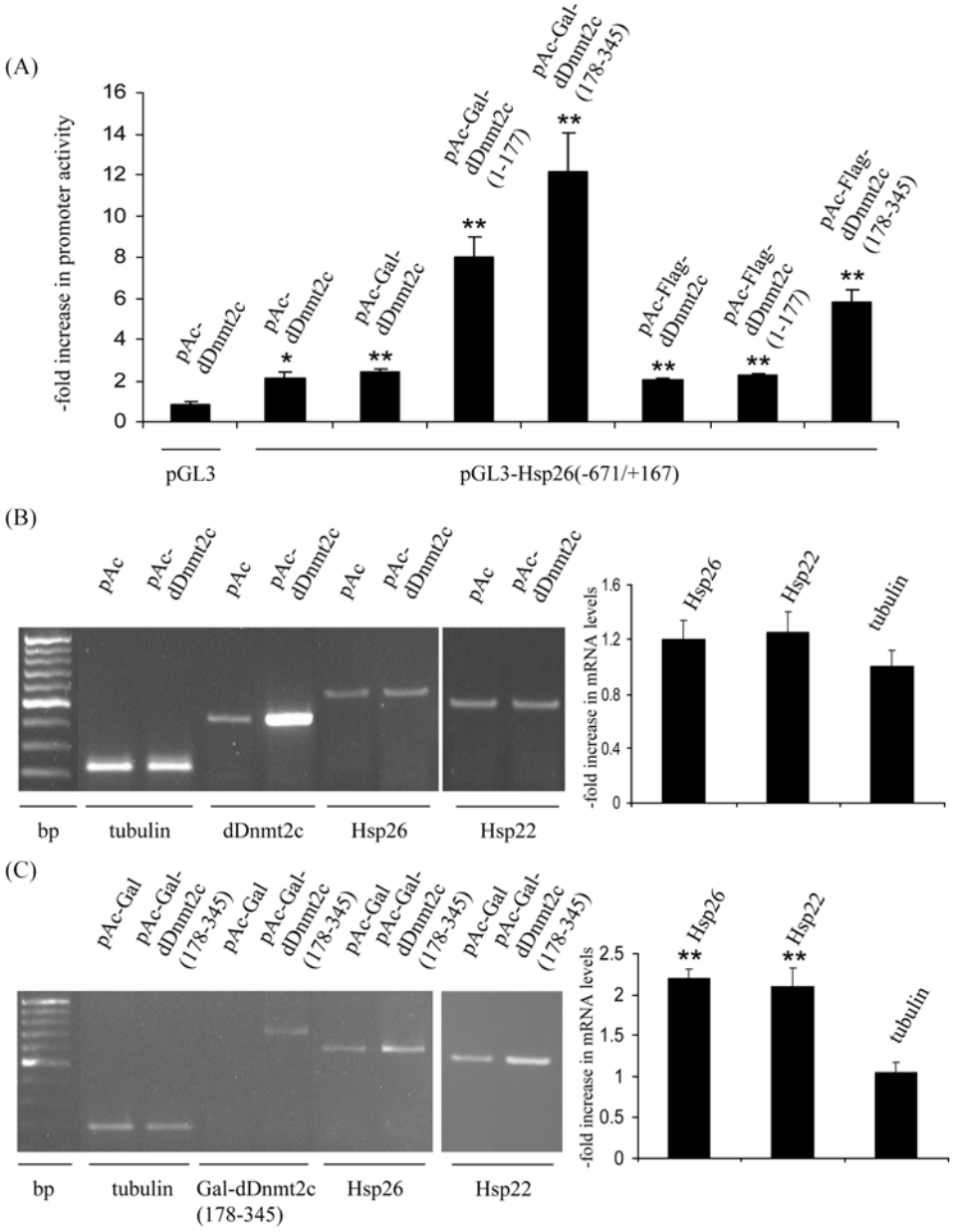

Fig. 1. The C-terminal half of dDnmt2c increases the level of endogenous Hsp26 mRNA *via* transcription. (A) dDnmt2c(178-345)-dependent activation of the Hsp26 promoter in S2 cells. The Hsp26 promoter construct, pGL3-Hsp26(-671/+167) was co-transfected with various expression vectors as indicated. The -fold increase in promoter activity represents the -fold increase in luciferase activity versus the respective control (co-transfected pAc5.1/V5-HisA, pAc-Gal or pAc-Flag). (B) No effect of overexpressed dDnmt2c on Hsp26 and Hsp22 mRNA levels in S2 cells. *Left panel*: RT-PCR was performed with RNA isolated from S2 cells transfected with pAc5.1/V5-HisA (pAc) or pAc-dDnmt2c. Specific primers were used to amplify dDnmt2c, Hsp26 and Hsp22. Beta-tubulin was amplified as an internal control. *Right panel*: respective Hsp26, Hsp22 and beta-tubulin signals were quantitated by densitometry. Values and their S.D. are the average of three independent experiments. (C) Increase in endogenous Hsp26 and Hsp22 transcripts by transiently expressed Gal4-dDnmt2c(178-345). *Left panel*: RT-PCR was performed with RNA isolated from S2 cells transfected with pAc-Gal or pAc-Gal-dDnmt2c(178-345). Specific primers were used to amplify Gal4-dDnmt2c, Hsp26 and Hsp22. *Right panel*: respective quantitated signals. Statistically significant differences between the transfected samples are indicated as $*P < 0.05$ and $**P < 0.01$.

dDnmt2c, pAc-dDnmt2c, and a luciferase reporter containing the Hsp26 promoter, pGL3-Hsp26(-671/+167), showed a significant, less than 2-fold stimulatory effect of dDnmt2c on Hsp26 promoter activity (Fig. 1A). Surprisingly, co-transfected expression vectors for the N-terminal and C-terminal halves of dDnmt2c fused to the Gal4 DNA-binding domain increased Hsp26 promoter activity 8- and 12-fold, respectively. To rule out an effect of the Gal4 DNA-binding

domain, co-transfections were repeated with plasmids encoding Flag-tagged dDnmt2c(1-177) and dDnmt2c(178-345). Again, the C-terminal half of dDnmt2c increased Hsp26 promoter activity more than 6-fold. Similar results were obtained with the promoter of the small heat shock protein, Hsp22 (data not shown). Semi-quantitative RT-PCR was used to see whether a transient expression of dDnmt2c or its C-terminal half, dDnmt2c(178-345), had any effect on endogenous Hsp26 or Hsp22 mRNA levels (Fig. 1B and 1C). Whereas dDnmt2c did not change the mRNA levels of Hsp22 and Hsp26, its C-terminal half significantly increased both mRNA levels approximately 2-fold.

The C-terminal half of dDnmt2c increases Hsp26 promoter activity *via* two heat shock elements, HSE6 and HSE7. Heat shock induction of *Drosophila* Hsp26 gene transcription requires mainly binding sites for heat shock factor (Hsf), *viz*. the heat shock elements (HSEs), HSE1, HSE2, HSE6 and HSE7 (Fig. 2A; Sandaltzopoulos *et al.*, 1995). Binding sites for GAGA factor play only a minor role in the heat shock response. As shown in Figure 2B, the effect of dDnmt2c and Gal4-dDnmt2c(178-345) on Hsp26 promoter activity was studied using a series of 5'-promoter deletions for co-transfections. Overexpressed dDnmt2c increased the luciferase activity of all Hsp26 constructs tested slightly, approximately 1.5- to 2-fold. In contrast, a sharp drop in dDnmt2c(178-345)-dependent stimulation was observed between -399 and -321 implying a fundamental role of this region in the induction of Hsp26 promoter activity. To see whether HSE6 and HSE7 found in this promoter region are involved in the dDnmt2c(178-345)-dependent stimulation, the core of each HSE (HSE6: $^{-346}$5'-GAA-3'$^{-344}$ to 5'-TCC-3'; HSE7: $^{-367}$5'-GAA-3'$^{-365}$ to 5'-TCC-3') was mutated in the construct, pGL3-Hsp26(-399/+167) and the resulting plasmids, pGL3-Hsp26(-399/+167)-HSE6m and pGL3-Hsp26(-399/+167)-HSE7m, were used in co-transfections. Both mutations reduced the dDnmt2c(178-345)-dependent increase in reporter gene activity significantly (from 8-fold to approximately 4-fold). The a globin promoter activity of a construct with two copies of the Hsp26 promoter region from -399 to -300 was induced by the C-terminus of dDnmt2c approximately 6-fold, whereas the α globin promoter in the construct pGL3-α did not respond at all (Fig. 2C). A simultaneous mutation of HSE6 and HSE7 almost completely abrogated the upregulation of α globin promoter activity. Mutations of HSE6 or HSE7, however, did not affect promoter activities of constructs co-transfected with an expression vector for dDnmt2c (Figs. 2B and 2C).

***Drosophila* Hsf is involved in the upregulation of Hsp26 promoter activity by the C-terminal half of dDnmt2c.** Marchler and Wu (Marchler and Wu, 2001) have shown that overexpression of Hsp40 (DROJ; DnaJ-1) in *Drosophila* S2 cells delayed the onset of the heat shock response. It is now well known that Hsp40 together with Hsp70 interact with Hsf to prevent it from binding to HSEs (Pirkkala *et al.*, 2001 and references therein). Data presented in Fig. 2 point towards the importance of an HSE-binding factor, probably Hsf itself in the induction of Hsp26 promoter activity by dDnmt2c(178-345) but not by dDnmt2c. To see whether Hsf is involved in this upregulation, pGL3-Hsp(-671/+167) was co-transfected with pAcGal-dDnmt2c(178-345) and increasing amounts of Hsp40 expression plasmid (Fig. 3A). Co-transfection of Hsp40 clearly reduced the dDnmt2c(178-345)-dependent increase in luciferase activity in a dose-dependent manner. However, Hsp40 had no effect on the transcriptional activation of a Gal4-containing reporter plasmid by a Gal4 DNA-binding domain fusion of the strong transcriptional activator, VP16 (Fig. 3B). Calderwood and colleagues (Wang *et al.*, 2004) recently demonstrated that a C-terminally truncated form of human heat shock factor 1, HSF1, competitively inhibited the activity of wild-type HSF1. A similar truncation of *Drosophila* Hsf was made, removing the C-terminal *trans*-activation domains but leaving DNA-binding and trimerization domains. The resulting expression plasmid, pAc-Myc-Hsf(1-537) was co-transfecetd with pAcGal-dDnmt2c(178-345) and an Hsp26 reporter plasmid (Fig. 3C). The co-transfection of the dominant negative form of Hsf counteracted the positive effect of dDnmt2c(178-345) on the Hsp26 promoter. Besides, Hsf(1-537) had no effect on the transcriptional activation of the activator, Gal-VP16 (Fig. 3D). Both Hsp40 and Hsf(1-537) co-transfection studies point towards the involvement of Hsf in the upregulation of Hsp26 promoter activity by the C-terminal half of dDnmt2c.

dDnmt2c interacts with Hsp70 *in vivo*. Under normal conditions, molecular chaperones such as Hsp40 and Hsp70 bind directly to the Hsf transactivation domain thereby preventing Hsf to form a trimeric form capable of activating heat shock protein genes (Morimoto, 1998; Santoro, 2000). Since the C-terminal half of dDnmt2c activates the Hsp26 promoter through heat shock elements, possibly *via* Hsf, it is conceivable that this protein may bind Hsf-inactivating chaperones releasing the Hsf monomers. That would eventually lead to formation of Hsf trimers able to activate transcription. Co-immunoprecipitations were performed to see whether wild-type dDnmt2c and its N- and C-terminal halves would interact with Hsp70 in S2 cells. For this purpose, S2 cells were transiently co-transfected with expression vectors for myc-tagged Hsp70Aa that belongs to a group of five Hsp70 genes in *D. melanogaster* (Leigh Brown and Irsh-Horowicz, 1981) and either Flag-tagged dDnmt2c, dDnmt2c (1-177) or dDnmt2c(178-345). Protein was crosslinked with DSP before cell lysis to identify any weak protein-protein interactions. In contrast to wild-type dDnmt2c, the N- and C-terminal halves showed a much lower expression level (Figs. 4A and 4B, right panels). Co-immunoprecipitations revealed an interaction between dDnmt2c and Hsp70Aa (Fig. 4A, left panel). Besides, an interaction between overexpressed dDnmt2c and myc-tagged Hsp40B1 functioning as an Hsp70 cochaperone (Fan *et al.*, 2003) was found (data not shown).

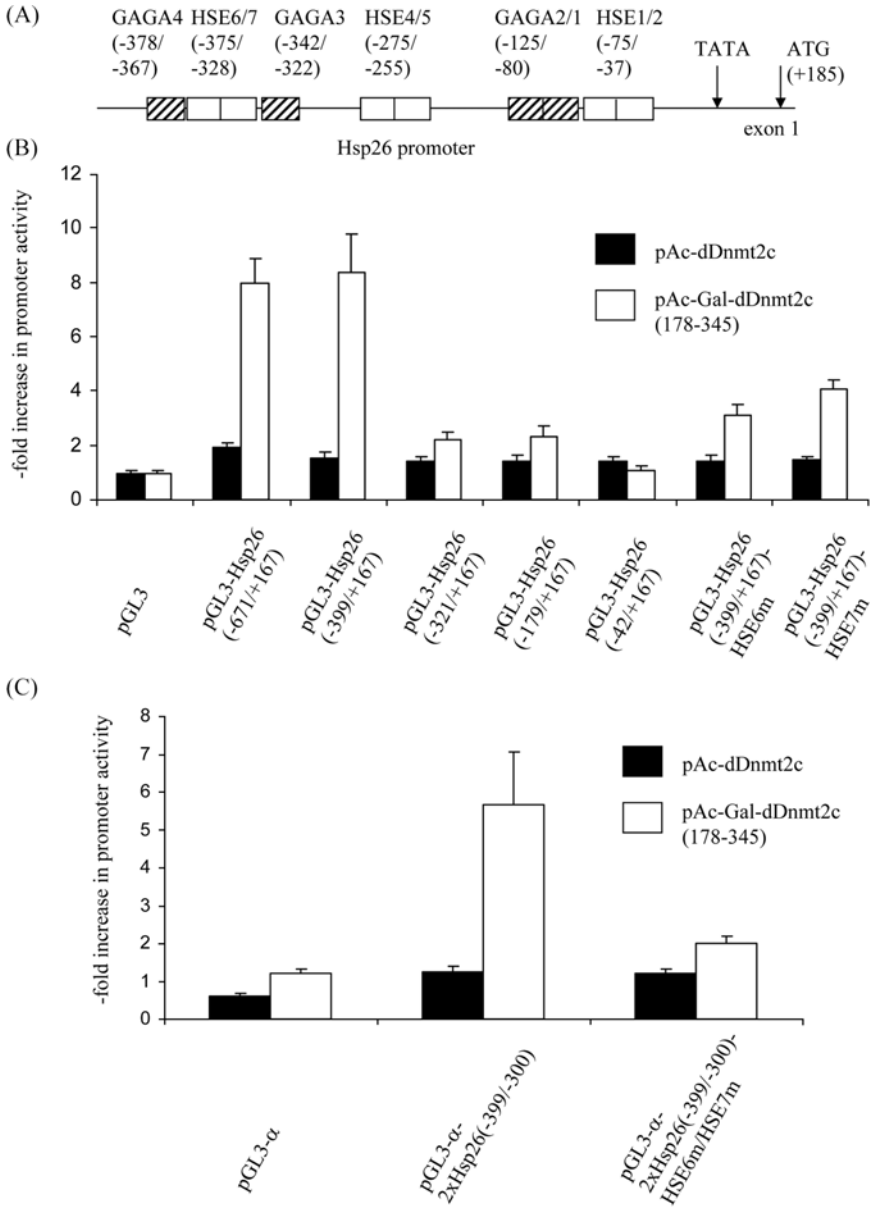

Fig. 2. HSE6 and HSE7 are responsible for the upregulation of Hsp26 promoter activity by the C-terminal half of dDnmt2c. (A) Schematic representation of the Hsp26 promoter with its binding sites for Hsf (HSEs) and GAGA factor (GAGA). (B) 5'-deletion promoter analysis. The effect of dDnmt2c and dDnmt2c(178-345) was analyzed by transient transfection of S2 cells with pAc-dDnmt2c or pAc-dDnmt2c(178-345) and the indicated Hsp26 promoter constructs. The plasmids, pGL3-Hsp26(-399/+167)-HSE6m and pGL3-Hsp26(-399/+167)-HSE7m, have triple point mutations in the core sequence of HSE6 and HSE7 (s. Materials and Methods). (C) The Hsp26 promoter from -399 to -300 can activate the heterologous α globin promoter upon co-transfection with an Gal4-dDnmt2c(178-345) expression vector. The construct, pGL3-α, and its Hsp26 promoter-containing derivatives were analyzed for responsiveness to co-transfected pAc-dDnmt2c or pAc-Gal-dDnmt2c(178-345). pGL3-α-2xHsp26(-399/-300)-HSE6m/HSE7m has triple point mutations in both HSE6 and HSE7.

Hsp70 and Hsp40 co-immunoprecipitations performed without a crosslinker gave identical results (data not shown). Interestingly, dDnmt2c(178-345) immunoprecipitated significantly more Hsp70Aa than the wild-type or the N-terminus implying that there are clearly more protein complexes containing dDnmt2c (178-345) and Hsp70Aa than there are complexes containing wild-type dDnmt2c and Hsp70Aa in the cell. Since endogenous levels of Hsp70 proteins are high, an interaction between dDnmt2c and Hsp70 protein could be detected (Fig. 4B, left panel). Despite its lower expression level, the C-terminally truncated dDnmt2c immunoprecipitated approximately 10-fold more endogenous Hsp70 compared to wild-type. Once again, the C-terminal half clearly formed more Hsp70-containing protein complexes than wild-type dDnmt2c.

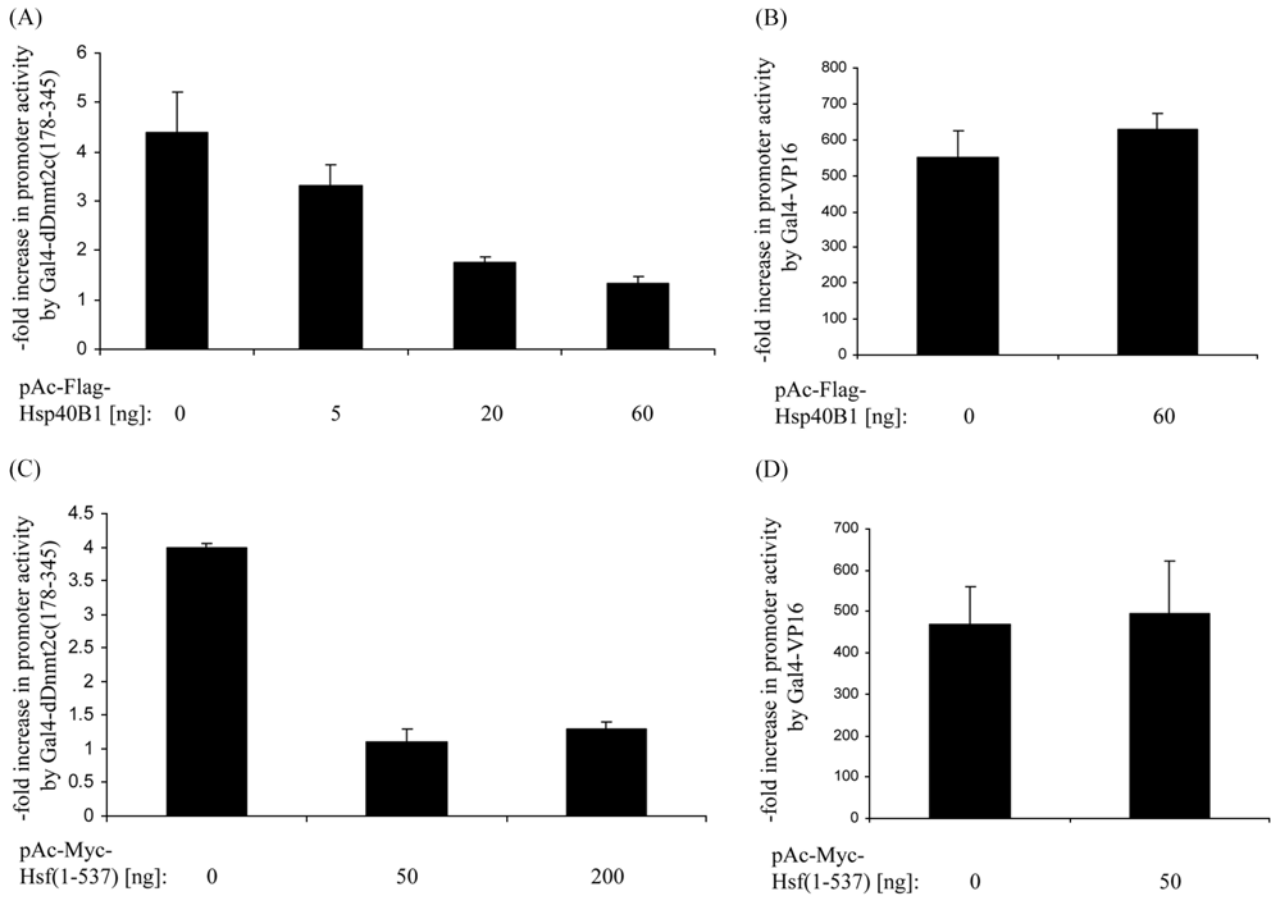

Fig. 3. Hsf is essential for the upregulation of Hsp26 promoter activity by the C-terminal half of dDnmt2c. (A) Hsp40 inhibits the ability of dDnmt2c(178-345) to *trans*-activate the Hsp26 promoter. S2 cells were transiently co-transfected with pGL3-Hsp26(-671/+167), pAc-Gal-dDnmt2c(178-345) and various amounts of pAc-Flag-Hsp40B1. The -fold increase in promoter activity represents the -fold increase in luciferase activity versus the respective control (co-transfected pAc-Gal and pAc-Flag). (B) Hsp40 does not affect the transcriptional activation of VP16. S2 cells were co-transfected with pGL3-TK-5G containing 5 Gal4-binding sites, pAc-Gal-VP16 and 0 or 60 ng of pAc-Flag-Hsp40B1. The -fold increase in promoter activity represents the -fold increase in luciferase activity versus the respective control (co-transfected pAc-Gal and pAc-Flag). (C) A dominant negative mutant of Hsf abolishes the upregulation of Hsp26 promoter activity by dDnmt2c(178-345). S2 cells were transiently co-transfected with pGL3-Hsp26(-671/+167), pAc-Gal-dDnmt2(178-345) and various amounts of pAc-Myc-Hsf(1-537). (D) Hsf(1-537) does not affect the transcriptional activation of VP16. S2 cells were co-transfected with pGL3-TK-5G, pAc-Gal-VP16 and 0 or 50 ng of pAc-Myc-Hsf(1-537).

Similar experiments were performed in 293T cells. For this purpose, 293T cells were transiently transfected with Flag-tagged hDnmt2a expression plasmid and used for immunoprecipitations after protein crosslinking. Western blot analysis revealed a weak signal for the Hsp70 immunoprecipitate of the Flag-hDnmt2a sample (Fig. 4C) implying an interaction between hDnmt2a and Hsp70 in 293T.

Discussion

Recently, Lin *et al.* (2004) reported that dDnmt2c is linked to the life span in fly. Whereas the intact gene is required for maintenance of the normal life span, overexpressed dDnmt2c caused longevity. The extended life span observed is most likely caused by increased levels of transcripts of several small heat shock proteins such as Hsp22, Hsp23 and Hsp26. This study has now been extended and a link between dDnmt2c and heat shock proteins in the *Drosophila* cell line S2 is reported. Surprisingly, only the C-terminus of dDnmt2c but not the full length protein can increase the endogenous mRNA levels of Hsp22 and Hsp26. Although it has to be shown that the carboxyterminus of dDnmt2c lacking the catalytic DNA methyltransferase domain IV indeed plays a biological role, it is tempting to speculate that dDnmt2c is involved in two independent physiological processes in fly, viz. DNA methylation and heat shock regulation. A life span test with transgenic flies overexpressing a catalytically inert dDnmt2c should unequivocally address this issue. As reported previously, a putative nuclear export signal is located close to the N-terminus of dDnmt2c (Hung *et al.*, 1999). This may explain the fact that dDnmt2c(178-345) is mainly found in the

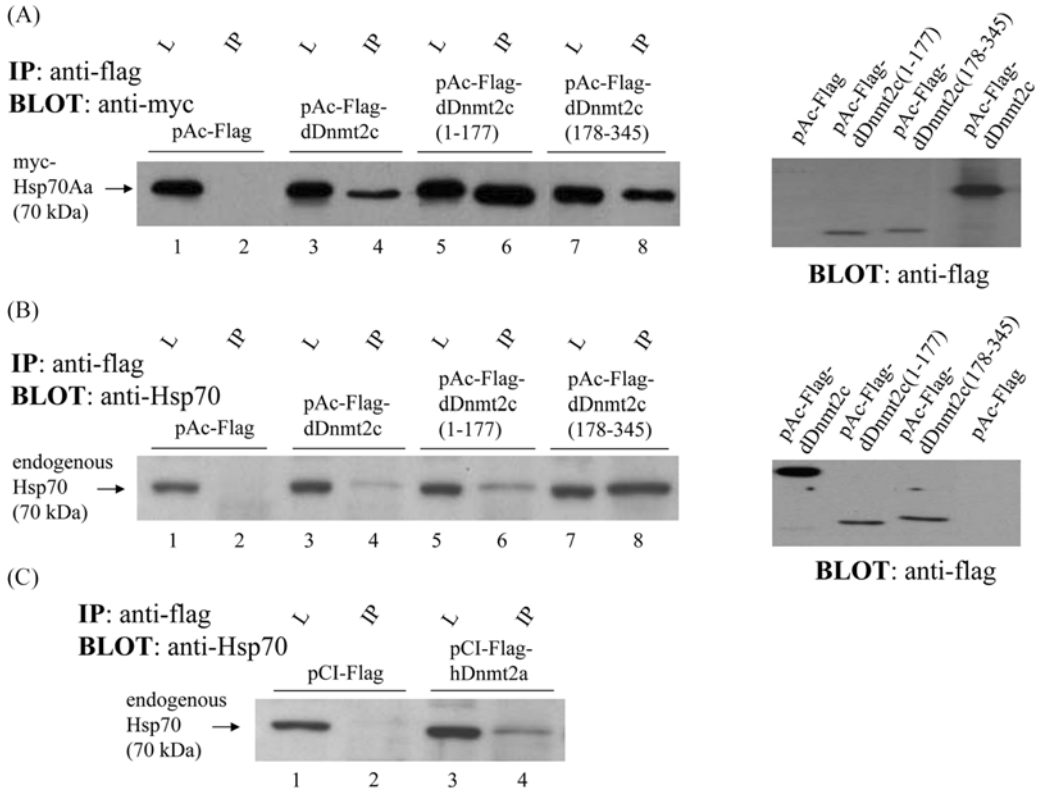

Fig. 4. Interaction of Dnmt2 with Hsp70 *in vivo*. (A) Interaction of dDnmt2c, dDnmt2c(1-177) and dDnmt2c(178-345) with overexpressed Hsp70Aa. *Left panel*: S2 cells were transiently co-transfected with pAc-Myc-Hsp70Aa and either pAc-Flag, pAc-Flag-dDnmt2c, pAc-Flag-dDnmt2c(1-177) or pAc-Flag-dDnmt2c(178-345). Cell lysates were immunoprecipitated with anti-Flag agarose. Total cell lysates (L) and immunoprecipitates (IP) were separated by SDS-PAGE, blotted and probed with anti-myc antibody. *Right panel*: Total cell lysates of co-transfected cells were separated by SDS-PAGE, blotted and probed with anti-Flag antiserum. (B) Interaction of dDnmt2c, dDnmt2c(1-177) and dDnmt2c(178-345) with endogenous Hsp70. *Left panel*: S2 cells were transiently transfected with either pAc-Flag, pAc-Flag-dDnmt2c, pAc-Flag-dDnmt2c(1-177) or pAc-Flag-dDnmt2c(178-345). Cell lysates were immunoprecipitated with anti-Flag agarose. Lysates and immunoprecipitates were separated by SDS-PAGE, blotted and probed with anti-Hsp70 antibody. *Right panel*: Lysates of cells were probed with anti-Flag antiserum. (C) Interaction of hDnmt2a with endogenous Hsp70. 293T cells were transfected with either pCI-Flag or pCI-Flag-hDnmt2a. Cell lysates were immunoprecipitated with anti-Flag agarose. Lysates and immunoprecipitates were probed with anti-Hsp70 antibody.

nucleus, whereas the wildtype protein is primarily found in the cytosol (Yu-Chiau Shyu, unpublished data). One may envisage several possibilities as to explain the increased transcription of Hsp26 by the C-terminal domain of dDnmt2c: (1) Similar to denatured protein during heat shock or other forms of stress, dDnmt2c(178-345) may bind Hsf-inactivating chaperones such as Hsp40 and Hsp70. They directly interact with the Hsf transactivation domain thereby preventing Hsf to form a trimeric form capable of activating heat shock protein genes under normal conditions. (2) Alternatively, dDnmt2c (178-345) might form a complex with Hsf that stimulates the nuclear entry and formation of transcriptionally active trimers of Hsf, similar to a phenomenon reported for c-myb and HSF3 (Kamano and Klempnauer, 1997; Kanei-Ishii *et al.*, 1997). However, no interactions between dDnmt2c or its C-terminal part with Hsf could be detected (data not shown). (3) The protein dDnmt2c(178-345) transactivates genes through direct or indirect binding to HSEs. This, however, is the least likely scenario since chromatin immunoprecipitations did not reveal any binding of flag-tagged dDnmt2c or dDnmt2c(178-345) to the promoter region of small heat shock protein genes (Shau-Ching Wen, unpublished data). Data presented clearly point towards scenario one. Observing approximately 10-fold more protein complexes containing dDnmt2c(178-345) and Hsp70 than complexes containing wild-type dDnmt2c and Hsp70, may explain why only the C-terminal part of dDnmt2c and not the wild-type protein can increase small heat shock protein mRNA levels *via* the above mechanism suggested. Also, preliminary immunofluorescence data (Yu-Chiau Shyu, unpublished data) support the first model. Increased Hsf levels in the nucleus are noticed in cells expressing dDnmt2c(178-345) compared to the control. In cells transfected with an expression vector for dDnmt2c, some nuclear entry of Hsf is seen but at a much lower level compared to cells with dDnmt2c(178-345). One may speculate that overexpressed dDnmt2c in fly could follow a similar mechanism. Indeed,

chromatin immunoprecipitations indicated increased binding of Hsf to the Hsp26 promoter in dDnmt2c-overexpressing fly embryos (Shau-Ching Wen, unpublished data). Currently, it is not clear why wild-type dDnmt2c increases mRNA levels of small heat shock proteins (sHsp) in fly but has no effect on respective mRNA levels in S2 where only the C-terminal half of dDnmt2c positively affects sHsp genes. One possibility might be that in S2 cells, the N-terminus of dDnmt2c somehow interferes with the binding of Hsp70 to the C-terminus, e.g. by cell line-specific protein(s) bound to the N-terminal part. Alternatively, partial degradation of the N-terminal part of dDnmt2c may occur in fly resulting in proteins with higher affinity for Hsp70. However, no shorter isoforms could be detected by Western blot analyses using S2 (data not shown) or fly (Kunert *et al.*, 2003; Lin *et al.*, 2004) overexpressing dDnmt2c. Currently, it is unclear whether the findings are attributable to a physiological function or just reflect partial misfolding of the C-terminally truncated dDnmt2c. Also, it can only be speculated whether Hsp70 is involved in Dnmt2 regulation or only in correct folding of Dnmt2 like of many other client proteins. However, the *in vivo* interaction data presented provide a plausible explanation why Dnmt2 overexpression results in an increase in small heat shock proteins in fly and Hsp70 in *Entamoeba histolytica*.

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