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H Granok
Washington University in St. Louis

B Leibovitch
Washington University in St. Louis

Sarah C.R. Elgin
Washington University in St. Louis, selgin@wustl.edu

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A heat-shock-activated cDNA encoding GAGA factor rescues some lethal mutations in the *Drosophila melanogaster Trithorax-like* gene

H. GRANOK†, B. A. LEIBOVITCH AND S. C. R. ELGIN*
Department of Biology, Campus Box 1229, Washington University, St Louis, MO 63130, USA

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Summary

GAGA factor is an important chromosomal protein involved in establishing specific nucleosome arrays and in regulating gene transcription in *Drosophila melanogaster*. We developed a transgenic system for controlled heat-shock-dependent overexpression of the GAGA factor 519 amino acid isoform (GAGA-519) *in vivo*. Efficient production of stable protein from these transgenes provided genetic rescue of a hypomorphic *Trithorax-like* (*Trl*) lethal allele to adulthood. Nevertheless, supplemental GAGA-519 did not suppress position effect variegation (PEV), a phenomenon commonly used to measure dosage effects of chromosomal proteins, nor did it rescue other lethal alleles of *Trl*. The results suggest requirements for the additional isoforms of GAGA factor, or for more precise regulation of synthesis, to carry out the diverse functions of this protein.

1. Introduction

It is well established that generation of an appropriate chromatin structure is important for regulation of eukaryotic gene expression (for reviews, see Felsenfeld, 1992; Wallrath *et al*., 1994; Wolffe, 1998). While in many cases chromatin remodelling is part of the activation process, in other cases genes appear to be 'pre-set' in a precise nucleosome array that leaves key regulatory elements in nucleosome-free regions. Such nucleosome-free sites are hypersensitive to cleavage by nucleases, and are often referred to as DNase I hypersensitive sites (DH sites) (Elgin, 1988; Wallrath *et al*., 1994). A critical component that appears to be involved in the formation of pre-set DH sites in *Drosophila melanogaster* is the abundant DNA binding protein, GAGA factor. GAGA factor has been shown to bind specific DNA regulatory elements of many *Drosophila* genes and has been localized *in vivo* to many sites along the chromosome arms and to the centromeric regions of metaphase chromosomes (reviewed in Granok *et al*., 1995; Wilkins & Lis, 1997; Farkas *et al*., 2000; see also Raff *et al*., 1995; Platero *et al*., 1998).

* Corresponding author. Tel: +1 (314) 935 5348. Fax: +1 (314) 935 5125. e-mail: selgin@biology.wustl.edu.
† Current address: Crossroads School, 500 DeBaliviere Ave, St. Louis, MO 63112, USA.

Lu *et al*. (1992, 1993) observed that GAGA factor binding sites 5′ of the *hsp26* transcription start site are necessary for the formation of the two DH sites found at this promoter; these DH sites encompass the heat shock regulatory elements. In the absence of the native GAGA factor binding sites, the DH sites are not formed and the gene is not heat-shock inducible. Further studies using the *hsp70* gene have shown that the GAGA factor binding sites are critical in establishing the normal transcription complex, which includes a paused molecule of RNA polymerase II at the promoter *in vivo* (Lee *et al*., 1992; Shopland *et al*., 1995; reviewed in Lis & Wu, 1993). The involvement of GAGA factor in chromatin structure determination is supported by studies using reconstituted chromatin templates *in vitro*. When purified GAGA factor is added to a reconstituted *hsp70* promoter (Tsukiyama *et al*., 1994) or *hsp26* promoter (Wall *et al*., 1995), nucleosome disruption is observed over and immediately adjacent to the GAGA factor binding sites.

GAGA factor is encoded by the gene *Trithorax-like* (*Trl*) (Farkas *et al*., 1994). *Trl* alleles have been identified both due to their effects on *Ultrabithorax* (*Ubx*) expression and due to their dominant enhancement of position effect variegation (PEV) (Dorn *et al*., 1993*a*, *b*; Farkas *et al*., 1994). PEV, the clonal inactivation of genes placed adjacent to heterochromatin following a chromosomal rearrangement,

has been proposed to involve changes in chromatin packaging (Locke *et al.*, 1988; Wallrath & Elgin, 1995; reviewed in Weiler & Wakimoto, 1995; Elgin, 1996). Flies homozygous for *Trl* mutations die mostly as third instar larvae (Farkas *et al.*, 1994). One allele, Trl^{13C}, is a functional hypomorph; flies homozygous for this mutation have reduced but detectable levels of GAGA factor and occasionally can survive under favourable conditions (Farkas *et al.*, 1994; Bhat *et al.*, 1996). Fertilized embryos produced from rare Trl^{13C}/Trl^{13C} females exhibit defects in the early mitotic divisions, including decondensed, fragmented and bridged chromosomes; specific effects on known target genes have also been observed (Bhat *et al.*, 1996).

Cloning and conceptual translation of GAGA factor cDNAs (Soeller *et al.*, 1993; Benyajati *et al.*, 1997) reveals that the protein contains a single zinc finger with an adjacent basic region; this domain is responsible for the high-affinity DNA binding to $(GA)_n$ sequences (Pedone *et al.*, 1996). In addition, GAGA factor contains a glutamine-rich carboxyl terminus, and an amino terminal BTB/POZ domain; both domains appear to be involved in protein–protein interactions (Zollman *et al.*, 1994; Bardwell & Treisman, 1994; Chen *et al.*, 1995; Agianian *et al.*, 1999; Wilkins & Lis, 1999; Katsani *et al.*, 1999; Espinás *et al.*, 2000; reviewed in Granok *et al.*, 1995; Wilkins & Lis, 1997; Farkas *et al.*, 2000). Northern and western blot assays show that GAGA factor is represented by multiple transcripts and multiple isoforms at all stages of development (Gilmour *et al.*, 1988; Biggin & Tjian, 1988; Soeller *et al.*, 1993; Benyajati *et al.*, 1997). The most prominent isoform, which is present at all developmental stages, is a 519 amino acid (aa) protein encoded by a 2·4 kb mRNA (GAGA-519). Recently, additional cDNAs for GAGA factor have been recovered that encode an alternate 581 aa isoform (GAGA-581) (Benyajati *et al.*, 1997). Some of the additional isoforms observed may represent post-translational modifications of the GAGA factors synthesized using these two transcripts.

We desired to establish a system that would allow the amount of GAGA factor in the fly to be experimentally manipulated. Supplemental GAGA factor could be used to study the developmental requirements for GAGA factor and the dynamic interplay among GAGA factor, histones, and other non-histone chromosomal proteins in setting chromatin structure. In this report we describe a gene system, hsp70.GAGA (hG), that utilizes a heat shock promoter to drive *in vivo* expression of a GAGA factor cDNA encoding GAGA-519. We show that these transgenic flies, under heat shock conditions, produced abundant and stable GAGA-519, and that overexpression of this transgene rescued Trl^{13C} homozygous flies from lethality.

2. Materials and methods

(i) Drosophila *strains*

All fly stocks were maintained on standard corn meal medium (Ashburner, 1989). The following stocks were used: *CyO*; TM2, Ubx^{130} *ry*/T(2:3), ap^{Xa} (from J. Lis, Cornell University); Trl^{62}/TM3, *Sb* $ry^{RK}e$ (from G. Reuter, Martin Luther University, Germany); Trl^{13C}/TM2, $Ubx^{130}ry$ and Trl^{R85}/TM3, *Sb Ser* (from F. Karch, University of Geneva, Switzerland); *w*; *hsp83*.GAGA and Trl^{R67}/TM6B, *Tb Hu e* (from A. Greenberg and P. Schedl, Princeton University); *y* w^{m4} (from J. Kennison, NIH); *red e* (from T. Breen, Southern Illinois University); Df(3R) e^{ddE}/TM2, $Ubx^{130}ry^{se}$ *red* e^{s} (from C. Nusslein-Volhard, Tübingen University, Germany). Additional stocks were either obtained from the *Drosophila* Stock Center (Bloomington, IN), or were constructed using standard genetic techniques. All balancer chromosomes used in this study are described in Lindsley & Zimm (1992).

(ii) *Plasmid construction*

All experimental manipulations of recombinant DNA were performed as described by Sambrook *et al.* (1989). The starting expression plasmid was pETGAGA, which contains the complete coding sequence of GAGA-519 (S. Alignay & J. T. Kadonaga, unpublished). A 1·9 kb *Xba*I/*Nhe*I fragment of pETGAGA (which contains the GAGA factor coding sequence, 41 bp of 5′ non-coding vector sequence, and 146 bp of 3′ non-coding vector sequence) was ligated into the *Xba*I site of pW8D44 (D. Johnson, unpublished). This recombinant plasmid was designated phsp70.GAGA. pW8D44 contains the *hsp70* promoter, the multiple cloning site of pW8 (Klemenz *et al.*, 1987), and approximately 2 kb of 3′ *hsp70* sequences, including the polyadenylation site and transcription termination site (Amin *et al.*, 1987). phsp70.GAGA was digested with *Sal*I and a 2·2 kb restriction fragment containing the *hsp70* promoter and the GAGA factor coding sequences was ligated into *Sal*I-digested Carnegie 20T (Xiao & Lis, 1988). The resulting plasmid was designated pCarhsp70.GAGA (phG; Fig. 1).

(iii) Drosophila *germline transformation*

Independent transformed lines (designated hG) were obtained following co-injection of phG and helper plasmid pπ25·7wc into ry^{506} embryos as described (Karess & Rubin, 1984; Spradling, 1986). The copy number and integrity of the transgenes were verified by Southern hybridization using probes for both GAGA factor and *ry* sequences (data not shown). The transformed lines were either made homozygous or

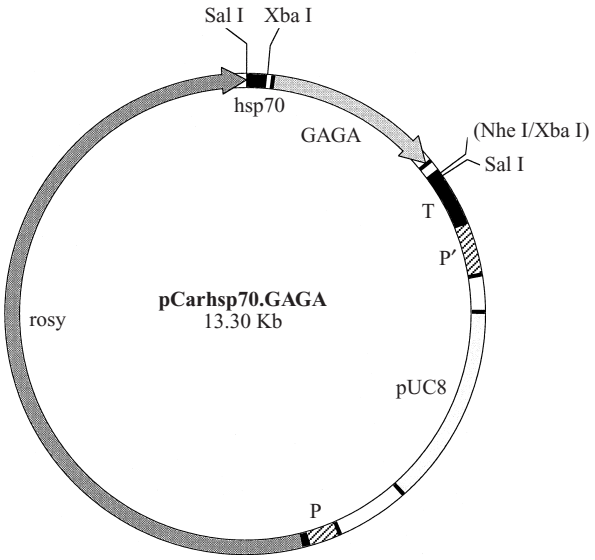

Fig. 1. Structure of pCarhsp70.GAGA (phG). This plasmid contains the complete GAGA factor coding sequence, with 41 bp of 5′ non-coding sequence and 146 bp of 3′ non-coding sequence from pET3a, under the transcriptional control of the *D. melanogaster hsp70* promoter. The *P*-element transformation vector is Car20T (Xiao & Lis, 1988). For clarity, only those restriction sites used in the construction are shown. For details of construction, see Materials and Methods.

the transgenes were maintained over the appropriate balancer chromosome.

(iv) *Antibody production and purification*

Recombinant GAGA-519 (rGAGA factor) was purified from *E. coli* by cation exchange chromatography as described (Lu *et al*., 1993). rGAGA factor was further purified by SDS PAGE, and the excised gel slice was used to immunize rats as described (Amero *et al*., 1988). Antibodies were purified from serum by precipitation with ammonium sulphate followed by batch chromatography using DEAE cellulose (Harlow & Lane, 1988).

(v) *Western blot analysis*

Nuclei were isolated from 6- to 18-hour-old embryos as described (Gilmour *et al*., 1988) and the proteins were size-separated by SDS PAGE. The proteins were transferred to nitrocellulose and the filters were blocked with 3% non-fat milk in phosphate-buffered saline. Blocked membranes were incubated with a 1:2000 dilution of anti-GAGA factor antibody in Tris-buffered saline, pH 7·4, 0·05% Tween-20 (TBST) containing 1% bovine serum albumin (BSA). After washing, the membranes were incubated for 45 min with alkaline-phosphatase-conjugated goat anti-rat antibodies (Cappel/Organon Teknika, Durham, NC) in TBST/1% BSA. The secondary antibody was detected using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) according to the manufacturer's instructions (Promega).

For analysis of heat-shock-induced expression, five flies carrying the hG transgene, or five ry^{506} control flies, were heat shocked in a 37 °C incubator for 1 h and then frozen in liquid nitrogen. The flies were then homogenized in 100 μl of SDS PAGE sample buffer and the homogenates were boiled in a water bath for 3 min, chilled on ice, and centrifuged for 1 min to precipitate insoluble material. Twenty microlitres of the resulting supernatants were loaded onto a 12% polyacrylamide SDS gel for electrophoresis and western analysis as described above. In experiments in which the amount of induced GAGA-519 was quantified, purified rGAGA factor was added to control samples prior to boiling.

(vi) *Genetic rescue of* Trl *alleles*

Three transgenes – hG2 and hG14 on the second chromosome, and hG22 on the X chromosome – were tested for genetic rescue of *Trl* lethality by constructing stocks with hG/*CyO*; *Trl*/TM3, *Sb ry e* or hG22; *Trl*/TM3, *Sb ry e*, where *Trl* represents each of the alleles studied: Trl^{62}, Trl^{13C}, Trl^{R85} or Trl^{R67}. The genotypes of these stocks were verified by genomic Southern blotting (data not shown). Trl^{62} was used to test for rescue of heteroallelic combinations by crossing hG22; hG2/*CyO*; Trl^{62}/TM3, *Sb ry e* females to males heterozygous for either Trl^{R85}, Trl^{R67} or Trl^{13C}. A heteroallelic survival test of Trl^{13C}/Trl^{R67} with hG2 was also performed. In all survival tests, vials were either maintained at room temperature or were heat shocked at 37 °C for 1 h per day. The presence of *Trl*/*Trl* progeny was assessed by the lack of dominant markers from the balancer chromosomes and was confirmed by Southern blot hybridization. Additional rescue tests were performed with two independent stocks in which Trl^{62} had been recombined onto a third chromosome marked with *red* and *e*. The genotypes of these recombinant stocks were verified by Southern blot hybridization. For larval rescue tests, Trl^{62}/Tb females were crossed with control Trl^{62}/Tb males, or with hG22; hG2/*CyO*; Trl^{62}/Tb or hG22; Trl^{62}/Tb males. Pupating homozygous Trl^{62} larvae (non-*Tubby* individuals) were counted and then removed from the vials over a period of 6 d. Between 200 and 300 pupae were scored for each cross.

(vii) *PEV characterization*

Male flies carrying the hG transgene, or ry^{506} controls, were crossed to $y\ w^{m4}$ females. Control flies were raised at 25 °C while a test population was heat shocked at 37 °C for 1 h each day until eclosion. After eclosion, young adult flies were kept at 18 °C until further use. Eye pigment extraction was performed as

previously described (Khesin & Leibovitch, 1978), using extracts from *y* w^{67c23} flies as a control. Five replicates were performed for each experiment.

3. RESULTS

(i) *Heat-shock-inducible expression of hG transgenes*

The *P*-element transformation plasmid, pCarhsp70.GAGA (phG) (Fig. 1), contains cDNA encoding the most abundant isoform of GAGA factor, GAGA-519. We recovered seven independent lines showing germline transmission of the ry^+ phenotype with a single, intact copy of the transgene. Western blot analysis was performed to verify that GAGA-519 is produced from the hG transgenes. Antibodies generated against purified, recombinant GAGA-519 (rGAGA factor) recognize a set of GAGA factor isoforms in embryo nuclei that range in size from 66 to 90 kDa (Fig. 2*A*). This pattern is consistent with that obtained using DNA affinity-purified GAGA factor from *D. melanogaster* embryo nuclei (Biggin & Tjian, 1988; Gilmour *et al.*, 1988).

Adult flies containing the hG transgene, or ry^{506} controls, were heat shocked for 1 h at 37 °C. As expected, low but detectable levels of endogenous GAGA-519 were observed in extracts from adult flies in the absence of heat shock (Fig. 2*B*). Following heat shock, the protein band migrating with a molecular weight of approximately 66 kDa was observed to give a much stronger signal in extracts from transgenic flies (Fig. 2*B*). The size of this band corresponds precisely to that of the purified rGAGA factor used as the antigen (compare with lanes including rGAGA factor). Using known amounts of rGAGA factor for comparison (Fig. 2*B*, rGAGA factor lanes), we estimate that approximately 80 ng of GAGA-519 is induced per fly (total body mass of 0·7 mg). This is approximately a 100-fold increase over the level of

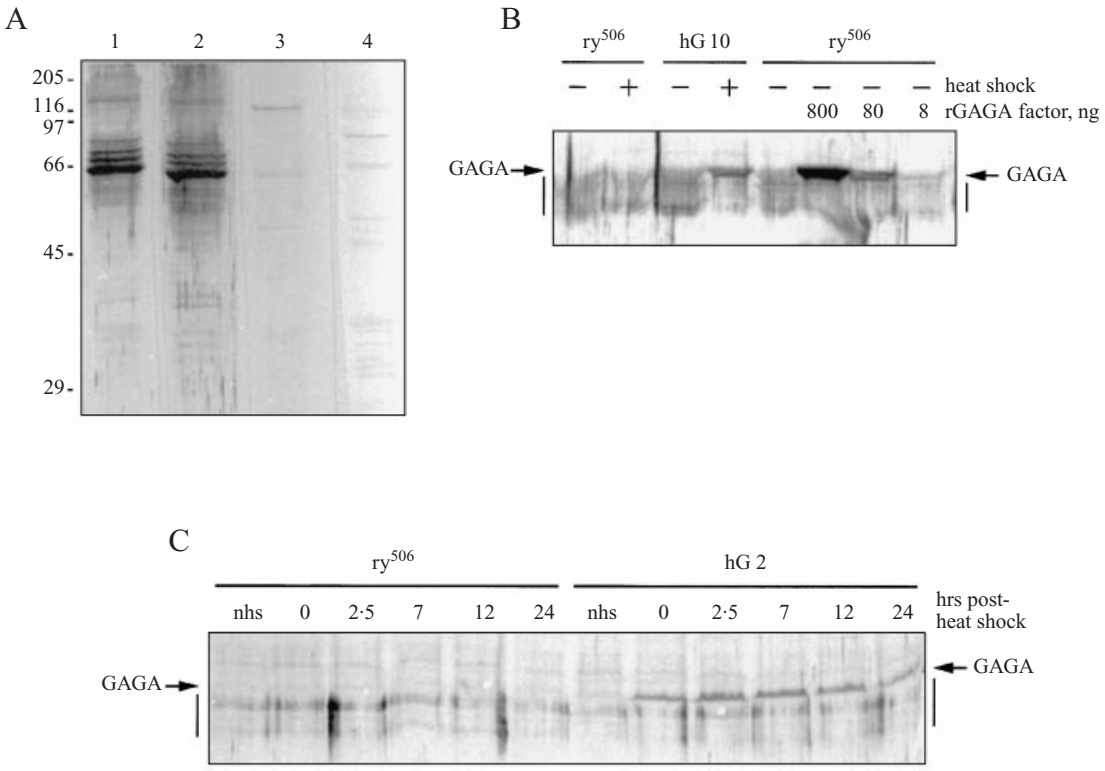

Fig. 2. GAGA-519 can be overexpressed in hG flies. (*A*) Characterization of the anti-GAGA factor antibodies. A western blot analysis of *D. melanogaster* embryo nuclear proteins was performed using antisera raised against purified recombinant GAGA-519 (rGAGA factor). Each of two antisera tested (lanes 1 and 2), but not preimmune sera (lanes 3 and 4), recognizes a set of proteins ranging in apparent molecular weight from 66 to 90 kDa. The positions of the molecular weight markers are indicated at the left. (*B*) Heat-shock-induced GAGA-519. Adult hG flies, or ry^{506} controls, were either heat shocked for 1 h or maintained at room temperature. Crude homogenates from single flies were fractionated by SDS PAGE and analysed by western blotting. In the blot shown (line hG10), the location of GAGA-519 is marked with an arrow. The vertical bars mark a set of constitutive, cross-reacting proteins that appear to be unrelated to GAGA factor. The bottom portion of the filter was probed using an antibody against actin-capping protein ß to verify equal loading in each well (data not shown). In the three lanes at the right of the blot, known amounts of rGAGA factor were mixed with homogenates from ry^{506} flies prior to SDS PAGE to provide a quantitative marker. (*C*) Stability of heat-shock-induced GAGA-519. hG2 flies, or ry^{506} controls, were heat shocked at 37 °C for 1 h and allowed to recover at 25 °C for the indicated lengths of time prior to homogenization and analysis by SDS PAGE and western blotting. The position of GAGA-519 is marked by an arrow. nhs, non-heat-shock control.

Table 1. *Rescue of* Trl *mutants to adulthood by hG transgenes*

Parental genotype	*Trl*/*Trl* flies	
	25 °C	hs 37 °C
Homoallelic stocks		
hG2/*CyO*; Trl^{62}/TM3	0 ($n > 1000$)	0 ($n > 1000$)
hG14/*CyO*; Trl^{62}/TM3	0	0
hG22; Trl^{62}/TM3	0	0
hG22; hG2/*CyO*; Trl^{62}/TM3	0	0
hG2/*CyO*; Trl^{R85}/TM3	0	0
hG22; Trl^{R85}/TM3	0	0
hG2/*CyO*; Trl^{13C}/TM3	0	4 ($n = 975$)
hG2/*CyO*; Trl^{13C}/TM3[a]	0	0 ($n > 2000$)
hG2/*CyO*; Trl^{R67}/TM3	0	0
hsp83.GAGA; Trl^{13C}/TM3	134 ($n = 1307$)	21 ($n = 162$)
hsp83.GAGA; Trl^{13C}/TM3[b]	118 ($n = 948$)	nd
Recombintant crosses[c]		
Trl^{62} *red e*/TM3 × hG2/*CyO*; Trl^{62}/TM3	nd	0 ($n > 3000$)
Trl^{62} *red e*/TM3 × hG22/Y; Trl^{62}/TM3	nd	0
hG22; Trl^{62}/TM3 × Trl^{62} *red e*/TM3	nd	0
hG22; hG2/*CyO*; Trl^{62}/TM3 × Trl^{62} *red e*/TM3	nd	0
Heteroallelic crosses[c]		
hG22; hG2/*CyO*; Trl^{62}/TM3 × Trl^{R85}/TM3, *Sb Ser*	nd	0 ($n > 1000$)
hG22; hG2/*CyO*; Trl^{62}/TM3 × Trl^{R67}/TM3	nd	0
hG22; hG2/*CyO*; Trl^{62}/TM3 × Trl^{13C}/TM2, *Ubx ry*	nd	0
Trl^{R67}/TM3 × hG2/*CyO*; Trl^{13C}/TM3	0 ($n = 1273$)	14 ($n = 1772$)

nd, not determined.
[a] Strain hG2/*CyO*;Trl^{13C}/TM3 after *c*. 2 yr of culture (showing loss of heat-shock-inducibility of the transgene).
[b] Strain hsp83.GAGA; Trl^{13C}/TM3 after *c*. 2 yr of culture.
[c] Maternal genotype appears first.

endogenous GAGA-519 in the adult fly. Similar results were obtained with all seven hG lines.

To measure the stability of the heat-shock-induced GAGA-519, hG2 flies were heat shocked for 1 h at 37 °C and were then allowed to recover at room temperature for different lengths of time prior to analysis by SDS PAGE and western blotting (Fig. 2*C*). Heat-shock-induced GAGA-519 was still easily detectable in these flies after 24 h. From these data we conclude that line hG2 produces large amounts of stable GAGA-519 following a 1 h heat shock. We therefore employed a heat shock protocol of 1 h per day at 37 °C in subsequent experiments to test the biological role of this isoform.

(ii) *Heat-shock-induced overexpression of GAGA-519 rescues some* Trl *mutants from lethality*

Trl mutant homozygotes die mostly as late third instar larvae (Farkas *et al*., 1994; Bhat *et al*., 1996). As a functional assay for the heat-shock-induced GAGA-519, we tested whether hG transgenes were able to rescue both homo- and heteroallelic *Trl* mutants from lethality. Four alleles of *Trithorax-like* were used in this analysis. Trl^{62} has a transposable element inserted 5′ of the transcription start site; Trl^{13C} has a transposable element inserted into an intron; Trl^{R67} has a deletion within the transcribed region; and Trl^{R85} has a large deletion of the 5′ transcribed and translated region (Farkas *et al*., 1994). We constructed stocks that carry an hG transgene on the first (hG22) or second chromosome (hG2, hG14) as well as balanced lethal *Trl* mutations on the third chromosome (Table 1). The progeny were allowed to develop at room temperature or were heat shocked 1 h per day to induce hG. At no time did we observe surviving adults among *Trl* homozygous flies that did not contain the transgene (thousands of individuals examined; data not shown). Several investigators (Farkas *et al*., 1994; Bhat *et al*., 1996) have reported the recovery of homozygous Trl^{13C} animals at a low frequency; however, under the growth conditions of our laboratory we have not observed any survivors. Of those stocks containing the transgene, Trl^{13C}/Trl^{13C} adults were recovered as 0·41 % of the total progeny from heat-shocked bottles (Table 1). The absence of a wild-type copy of *Trl* on the third chromosome was confirmed by genomic Southern blots of the DNA from the rescued animals (data not shown). The number of rescued flies was much lower than the theoretically expected value of 33 %.

In contrast to the results with Trl^{13C}, we were unable to obtain genetic rescue of animals homozygous for Trl^{62}, Trl^{R67} or Trl^{R85} (Table 1, homoallelic

stocks). To eliminate the possibility that the *Trl* mutant chromosomes contain additional lethal mutations that are preventing the recovery of homozygous individuals, we examined heteroallelic combinations; in addition, we recovered lines with Trl^{62} recombined onto an unrelated third chromosome marked with *red* and *e*. Two such independent recombinant lines with Trl^{62} were tested with hG2 or hG22; however, we again failed to observe homozygous Trl^{62} animals (Table 1, recombinant crosses). Trl^{62}/TM3, *Sb ry* females carrying two copies of hG were crossed to males heterozygous for other *Trl* alleles. No *Trl*/*Trl* animals were observed in the progeny, indicating a lack of complementation among these *Trl* alleles and with the hG transgene (Table 1, heteroallelic crosses). However, when Trl^{R67} females were crossed to Trl^{13C} males carrying the hG transgene, Trl^{13C}/Trl^{R67} adults were recovered as 0·79% of the progeny under heat shock conditions (*c.* 5% of the theoretically expected number; Table 1). Trl^{13C}/Trl^{13C} and Trl^{R67}/Trl^{13C} rescued adults can survive at room temperature no longer than 3 d and are sterile. From these data we conclude that GAGA-519 produced from hG rescues only hypomorphic Trl^{13C} mutants.

Trl^{62} homozygotes normally die as late third instar larvae, although a small number pupate. Therefore, we examined whether one or two copies of hG could increase the number of homozygous Trl^{62} pupae. For this assay, the Trl^{62} allele was balanced over TM6B, *Tb* to facilitate scoring in larvae and pupae. In control crosses, with and without heat shock treatment (37 °C, 1 h per day), homozygous Trl^{62} pupae appeared on the second day following pupation of their Trl^{62}/Tb siblings. After 4 d, approximately 4% of the total pupae were homozygous Trl^{62} animals. Trl^{62} homozygotes carrying two copies of hG pupated earlier than larvae lacking the hG transgenes. The total percentage of homozygous Trl^{62} pupae carrying hG22 and hG2 on the fourth day was approximately 7% without heat shock and was increased to 12% with heat shock treatment. Similar results were obtained with Trl^{62}/TM6B, *Tb* larvae carrying hG22 alone. Eclosion of homozygous animals was not observed. From these data we conclude that although heat-shock-induced GAGA-519 does not rescue homozygous Trl^{62} flies to adulthood, the additional protein does shift the lethal phase.

Interestingly, after the stocks carrying the hG transgene (with and without *Trl* mutations) had been propagated for *c.* 2 y, the lines lost their ability to synthesize high levels of additional GAGA-519 in response to heat shock (data not shown). Further, these transgenic lines were no longer able to rescue flies homozygous for Trl^{13C}, indicating a loss of inducible GAGA-519 activity (Table 1). We have also tested the efficiency of rescue by transgene hsp83.GAGA, in which a GAGA-519 cDNA has been placed under the control of the *hsp83* promoter (Greenberg & Schedl, 1999). Initially, we obtained rescue of Trl^{13C} homozygotes by hsp83.GAGA at a frequency of 10·3%, even in the absence of heat shock (Table 1, hsp83.GAGA; Trl^{13C}/TM3). In a rescue experiment 2 years later, 12·4% of the progeny were homozygous for the Trl^{13C} allele. This indicates that, unlike hsp70.GAGA, the efficiency of rescue by this transgene was unaffected by prolonged culture.

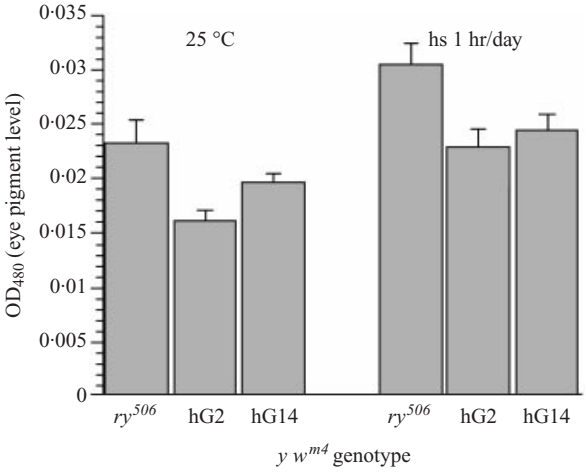

Fig. 3. Overexpression of GAGA-519 does not suppress PEV. Eye pigment levels were measured in $y\ w^{m4}$ males carrying hG2, hG14 or a wild-type chromosome from the ry^{506} injection stock. The flies were either maintained at room temperature or were subjected to a 37 °C heat shock for 1 h each day until eclosion. The mean value and standard deviation for eye pigment levels from five parallel extractions are shown for each line.

(iii) *Expression of hG does not suppress PEV*

Several *Trl* alleles have been shown to act as dominant enhancers of position effect variegation (PEV) (Dorn *et al.*, 1993*a*, *b*; Farkas *et al.*, 1994). We have also observed strong enhancement of PEV by Trl^{R85} using different w^{m4} stocks (data not shown). Several genes affecting PEV have been observed to show antipodal dosage effects, e.g., if one dose of the gene acts as an enhancer of PEV, three doses act as a suppressor (Wustmann *et al.*, 1989; Sinclair *et al.*, 1992; Eissenberg *et al.*, 1992). Therefore we examined whether or not overexpression of GAGA-519 might suppress PEV of the w^{m4} allele. As expected, the results showed that elevated temperature *per se* suppresses PEV (Spofford, 1976; Weiler & Wakimoto, 1995) (Fig. 3). The w^{m4}; ry^{506} flies subjected to heat shock showed a 30% increase in eye pigmentation. However, neither hG2 nor hG14 suppressed PEV relative to the ry^{506} control. Under non-heat-shock conditions, the hG chromosome may have slightly enhanced eye pigment variegation, reducing levels approximately 20%. Elevating the growth temperature of the w^{m4}; hG2 and w^{m4}; hG14 flies suppressed

PEV by 38% and 20%, respectively. This level of suppression was comparable to that obtained by elevating the growth temperature of the control flies.

4. Discussion

Using a cDNA transgene to produce GAGA-519 in a heat-shock-dependent fashion, we have demonstrated genetic rescue of flies with the Trl^{13C} allele (Table 1). Other alleles could not be rescued; however, the lethal phase of Trl^{62} homozygous larvae was shifted to later stages. The Trl^{13C} allele is a hypomorph, with decreased amounts of GAGA factor present in the flies. Under our laboratory conditions, heat shock induction of the transgene was essential for rescue. This result is consistent with the observation that the loss of heat-shock inducible GAGA-519 synthesis following prolonged culture (*c*. 2 yr) of the hG flies was accompanied by a loss of heat-shock-inducible rescue.

Several transcripts are produced from the *Trl* locus (Soeller *et al*., 1993; Farkas *et al*., 1994), and some of these transcripts are alternately spliced to encode different isoforms of GAGA factor. GAGA-519 and GAGA-581 differ in the glutamine-rich C-terminal domain (Benyajati *et al*., 1997). No major differences have been reported concerning the behaviour of these isoforms *in vitro* (Benyajati *et al*., 1997). However, western blots specific for GAGA-519 or GAGA-581 do show different developmental and tissue-specific profiles (Benyajati *et al*., 1997), indicating specific regulation in their synthesis and suggesting specificity in function. That the two isoforms are functionally different *in vivo* is further suggested by experiments with hsp83.GAGA cDNA transgenes, encoding either the 519 aa or 581 aa isoforms expressed using the *Drosophila hsp83* gene promoter. The hsp83.GAGA-519 transgene is more effective than the hsp83.GAGA-581 transgene in reversing the *Ubx* phenotype and in preventing sterility, although the two transgenes rescue flies from lethality with similar efficiency (Greenberg & Schedl, 1999; A. Greenberg & P. Schedl, personal communication).

The results suggest that other GAGA factor isoforms, in addition to GAGA-519, may be required for viability. Given the genomic changes of the mutations (Farkas *et al*., 1994), one can infer that Trl^{62}, Trl^{R67} and Trl^{R85} may eliminate several or all of the known transcripts, whereas some production of all isoforms might persist in Trl^{13C} (having a *P*-element insertion into an intron). In fact, Bhat *et al*. (1996) have shown that all the GAGA factor isoforms can be observed in ovarian tissue from Trl^{13C} homozygotes, although the levels of these proteins appear to be reduced compared with wild-type flies. The production of GAGA-519 from hG presumably supplies critical amounts of that isoform, allowing rescue of some Trl^{13C} homozygotes. However, hG apparently fails to compensate for the reduction or loss of other GAGA factor isoforms inferred to occur in response to other *Trl* mutations.

Alternatively, the amount or timing of synthesis of GAGA-519 from the hG transgene might be insufficient for the needs of the animal and may, in fact, be deleterious. The ability of GAGA factor *in vitro* to protect weak minimal GAG binding sites, as well as the size of the DNase I footprint on natural promoters, is concentration dependent (Katsani *et al*., 1999). The 100-fold increase in GAGA-519 levels following heat shock of hG lines may result in the misexpression of many genes. In addition, overexpression of GAGA-519 might cause inappropriate interactions between GAGA-519 and other nuclear proteins. Numerous studies have shown that protein–protein interactions are mediated by GAGA factor's BTB/POZ domain and by the glutamine-rich C-terminal domain (Bardwell & Treisman, 1994; Chen *et al*., 1995; Espinás *et al*., 1999; Katsani *et al*., 1999; Wilkins & Lis, 1999). GAGA factor from *D. melanogaster* embryo nuclei is recovered within the void volume following gel filtration chromatography (Gilmour *et al*., 1989; H. Granok & S. C. R. Elgin, unpublished observations). This indicates that GAGA factor is part of a large multiprotein complex and does indeed participate in protein–protein interactions *in vivo*. Thus, overproduction of GAGA-519 might improperly change the activity of many genes by both direct and indirect mechanisms.

Support for this possibility comes from comparing the results obtained using the hsp70.GAGA transgenic lines described above with results obtained using the hsp83.GAGA transgenic line (Table 1). The hG transgenes produced a 100-fold increase in GAGA-519 levels following heat shock. Although the hsp83.GAGA transgene does not produce a measurable change in GAGA-519 levels, even upon heat shock (A. Greenberg & P. Schedl, personal communication; B. Leibovitch & S. C. R. Elgin, unpublished observations), this transgene provides 25-fold better survival of both Trl^{13C}/Trl^{13C} and Trl^{13C}/Trl^{R67} flies (Table 1) than does the hsp70.GAGA transgene. A negative impact from excess GAGA-519 might contribute to the observed loss of activity in the hG lines upon prolonged culture; such loss is not observed with the hsp83.GAGA line (Table 1). Clearly, the hsp83.GAGA transgene is more effective in rescue experiments than the hsp70.GAGA transgene, due either to the amount of GAGA-519 produced or to the pattern of expression.

The results shown in Fig. 3 indicate that supplemental GAGA-519 does not suppress PEV. It is possible that GAGA factor is already present above stoichiometric amounts in wild-type flies, so no suppressive effects on PEV are observed upon over-

expression of GAGA-519. Alternatively, the lack of effect of supplemental GAGA-519 on PEV may simply be the consequence of misexpression of this protein, as mentioned above. In particular, supplemental GAGA-519 might be sequestered in inclusion bodies (Agianian *et al*., 1999) rather than forming productive complexes within chromatin.

We conclude that while artificial expression of the 519 aa isoform of GAGA factor is sufficient to compensate for a partial loss of the protein *in vivo*, it is not sufficient to perform all the required functions of this important protein. Further, *D. melanogaster* appears to be sensitive to the level of GAGA-519 produced; whether this reflects the total amount of protein, the ratio of isoforms, or both, is not yet resolved. Modifications of the expression system or of the expressed products may allow for the dissection of some of these requirements.

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