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GAGA Factor Isoforms Have Distinct but Overlapping Functions In Vivo

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The *Drosophila melanogaster* GAGA factor (encoded by the *Trithorax-like* [*Trl*] gene) is required for correct chromatin architecture at diverse chromosomal sites. The *Trl* gene encodes two alternatively spliced isoforms of the GAGA factor (GAGA-519 and GAGA-581) that are identical except for the length and sequence of the C-terminal glutamine-rich (Q) domain. In vitro and tissue culture experiments failed to find any functional difference between the two isoforms. We made a set of transgenes that constitutively express cDNAs coding for either of the isoforms with the goal of elucidating their roles in vivo. Phenotypic analysis of the transgenes in *Trl* mutant background led us to the conclusion that GAGA-519 and GAGA-581 perform different, albeit largely overlapping, functions. We also expressed a fusion protein with LacZ disrupting the Q domain of GAGA-519. This LacZ fusion protein compensated for the loss of wild-type GAGA factor to a surprisingly large extent. This suggests that the Q domain either is not required for the essential functions performed by the GAGA protein or is exclusively used for tetramer formation. These results are inconsistent with a major role of the Q domain in chromatin remodeling or transcriptional activation. We also found that GAGA-LacZ was able to associate with sites not normally occupied by the GAGA factor, pointing to a role of the Q domain in binding site choice in vivo.

The packaging of DNA into chromatin restricts its accessibility and interferes with many DNA-dependent processes such as replication and transcription (11). To circumvent this problem of accessibility, regions of chromatin that are nucleosome free and hypersensitive to low levels of nucleases (e.g., DNase I) are established (39). One protein that seems to have an important role in generating and/or maintaining nucleosome-free regions of chromatin is the *Drosophila melanogaster* GAGA factor (6, 16, 17, 31, 38). GAGA binding sites are found in the promoters and enhancers of many *Drosophila* genes including *hsp70*, *hsp26*, *fushi tarazu* (*ftz*), *engrailed* (*en*), and the bithorax complex (BX-C) gene *Ultrabithorax* (*Ubx*) (16, 18, 30, 38). In in vitro chromatin assembly experiments, the GAGA factor cooperates with the ATP-dependent nucleosome-remodeling factor to displace nucleosomes from promoters containing GAGA binding sites. In in vivo transgene assays, mutations in the *hsp70* and *hsp26* GAGA binding sites interfere with the formation of nucleosome-free regions of chromatin over each promoter and reduce transcriptional activity (21, 29). The role of the GAGA factor is not, however, limited to promoters and enhancers. GAGA binding sites are also present in Polycomb response elements (the *iab-7* PRE and a *bxd* PRE) and a domain boundary (*Fab-7*) from BX-C (18, 19). GAGA protein associates with these BX-C regulatory elements in vivo, and the GAGA sites in the PREs are critical for *Polycomb*-dependent silencing (19, 22). Finally, in diploid nuclei the GAGA factor localizes not only to euchromatin but also to heterochromatin, where it is thought to bind to the $(AAGAG)_n$ satellite DNA (26, 27). This heterochromatic localization is particularly evident during the rapid nuclear divisions in precellular blastoderm embryos.

Mutations in the gene encoding the GAGA factor, *Trithorax-like* (*Trl*), have been isolated (4, 12). The original *Trl* allele, Trl^{13C}, is a weak hypomorph and has reduced viability. Like mutations in other *Trithorax* group genes, it dominantly enhances the haltere-to-wing transformations observed in animals heterozygous for a *Ubx* mutation. A presumed null allele, Trl^{R67}, was generated by the imprecise excision of the Trl^{13C} P-element (12). There also are two ethyl methanesulfonate (EMS) alleles that have properties quite similar to Trl^{R67}. *Trl* is an essential gene, and animals homozygous for Trl^{R67} or either of the EMS alleles die before they reach the third larval instar. On the other hand, when Trl^{R67} or the EMS mutants are combined with the hypomorphic Trl^{13C} allele, a few of the animals survive to adulthood.

Since *Trl* is essential for viability, adult phenotypes can be studied only by using adult-viable allelic combinations. Flies homozygous for the hypomorphic Trl^{13C} mutation have homeotic transformations from the sixth abdominal segment to the fifth and are female sterile (12). The abdominal transformations indicate that *Trl* is required for the proper regulation of *Abdominal B* (*Abd-B*), a gene in the bithorax complex. The female sterility can be attributed to two different defects (4). First, Trl^{13C} females produce many fewer eggs than do wild-type females. Second, fewer than 10% of the embryos from Trl^{13C} mothers hatch even when fertilized by wild-type males. Only very little GAGA protein is expressed in embryos from Trl^{13C} mothers, and more than 70% do not develop beyond the cellular blastoderm stage. This early embryonic arrest is due to disruptions in the nuclear division cycles during cycles 10 to 14 after the nuclei have migrated to the surface of the embryo. Unlike what is observed for the wild type, the nuclear division

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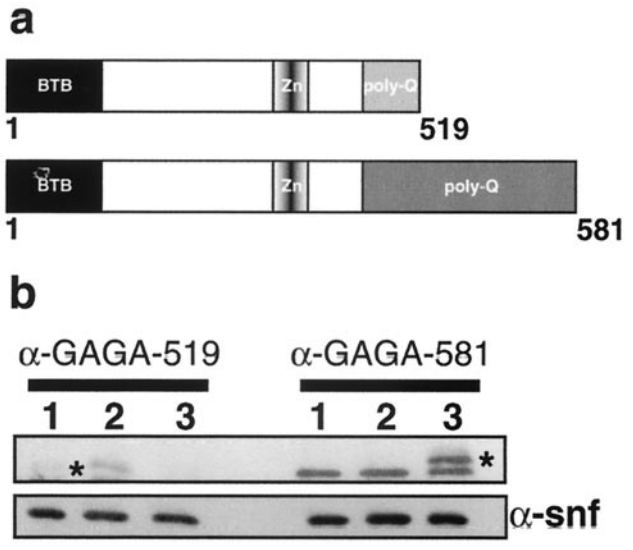


FIG. 1. (a) Schematic representation of the GAGA isoform constructs used in this study. Both transgenes are driven by the *hsp83* promoter. (b) Western blot showing expression of the *hsp83:GAGA-519* and *hsp83:GAGA-581* transgenes. Lanes: 1, embryos from a cross of w^1 males and females; 2, embryos from a cross of 4X*hsp83:GAGA-519*; $Trl^{81.1}/Trl^{109.2}$ males and females; 3, embryos from a cross of 4X*hsp83:GAGA-581*; $Trl^{R67}/Trl^{109.2}$ males and females. The first three lanes (left half of the gel) were probed with the antibody against GAGA-519 (α-GAGA-519). The weak band corresponding to the GAGA-519 protein in lane 1 is indicated with a star. The right half of the gel was probed with the GAGA-581 antibody (α-GAGA-581). This antibody recognizes a nonspecific band that appears in all three lanes. The band corresponding to the GAGA-581 protein is indicated with a star. α-Snf antibody was used as a loading control.

cycles in Trl^{13C} embryos are asynchronous and a range of mitotic defects is observed. These defects include incomplete chromosome condensation, asymmetric chromosome segregation, severe chromosome fragmentation, and the loss of nuclei from the surface. It is thought that abnormalities in the chromatin organization of the heterochromatic $(AAGAG)_n$ satellite DNA are responsible for the mitotic defects. While high levels of GAGA are associated with centromeric heterochromatin in wild-type embryos, there is little or no heterochromatic protein in most Trl^{13C} embryos (4). The few embryos that successfully cellularize and begin gastrulation display defects in expression of genes such as *ftz* and *en* that have GAGA binding sites in their regulatory regions (4). These defects are typically seen in nuclei that have little or no GAGA protein.

GAGA is synthesized in sufficient quantities from maternally deposited mRNA to sustain development through embryogenesis. However, maternal message is not the only source of GAGA in the embryo, and roughly midway through gastrulation zygotic *Trl* transcripts can be detected. In addition to the mRNAs encoding the GAGA-519 isoform, alternatively spliced transcripts encoding a second major GAGA isoform, GAGA-581, are expressed.

The GAGA-519 and GAGA-581 proteins have three distinct domains (Fig. 1a). The first two domains are present in both proteins, while the C-terminal domains, which are encoded by alternative 3′ exons, are unique. The N-terminal 120 amino acids (aa) comprise a so-called BTB/POZ (Bric a brac, Tramtrack, Broad-complex/Poxvirus, Zinc finger) domain. The BTB/POZ domain is found in a variety of proteins from yeast to human (2, 10, 15, 28). The central part of the protein contains a single zinc finger flanked by two small basic regions. The zinc finger and flanking basic regions are responsible for the DNA binding activity of the GAGA protein (24, 25). The C-terminal Q domains of the GAGA-519 and the GAGA-581 proteins are both highly glutamine rich, but they have distinct sequences and lengths (3).

While there are already some indications as to likely functions of the BTB/POZ and Zn-finger domains, the role of the C-terminal Q domains is not well understood. Chromatin assembly experiments indicate that the Q domains are dispensable for chromatin remodeling at least in vitro (1). Although the Q domain of the GAGA-519 protein seems to be important for transcriptional activation in transient-transfection assays (33), it is not at all certain whether the ability to affect transcription in these assays (3, 30, 33) is relevant to GAGA function in flies. Perhaps the most compelling evidence that the Q domain may be important comes from the studies of Wilkins and Lis (37). They showed that while the 519 Q domain is not required for DNA binding, it does alter the DNA binding properties of the protein. The 519 Q domain can itself interact with single-stranded DNA and may promote DNA distortion upon GAGA factor binding. In addition, the 519 Q domain mediates the tetramerization of the intact GAGA factor and in isolation can form multimeric complexes (1, 37).

How these activities of the 519 Q domain relate to the in vivo functions of the GAGA factor is not known. Moreover, it is not at all clear why there are two GAGA isoforms with completely different Q domains. The GAGA-519 and GAGA-581 proteins behave indistinguishably in in vitro DNA binding assays and in tissue culture transient-transfection experiments, and they colocalize in polytene chromosomes (3). On the other hand, the striking conservation of the two isoforms between distantly related species of fruit flies (20) suggests that these isoforms may have distinct functions. This possibility is supported by the differences in the stage- and tissue-specific patterns of expression of the GAGA-519 and GAGA-581 proteins (3).

In the studies reported here we have used rescue constructs to examine whether the GAGA-519 and GAGA-581 are functionally equivalent in vivo. We find that GAGA-519 and GAGA-581 have overlapping but not identical functions. To further elucidate the role of the Q domain, we replaced it with the bacterial protein β-galactosidase. Surprisingly, this GAGA-LacZ fusion protein has partial function, suggesting that the Q domain may not have a major role in chromatin remodeling or gene regulation.

MATERIALS AND METHODS

Fly stocks. Trl^{13C} is a P-element-induced allele (the P-element is located in the first intron of the gene) (12). Trl^{R67} (12) was generated by excision of the P-element that generated the Trl^{13C} allele. This mutation has sequences deleted from the 5′ half of the Trl transcription unit. $Trl^{81.1}$ and $Trl^{109.2}$ are independently isolated EMS-induced alleles (A. J. Greenberg, unpublished data).

All crosses were performed in w^1 background using the TM6b, *Tb Sb Hu e* balancer chromosome to ensure consistency of genetic background. Some crosses were repeated using the TM3, *Ser e* balancer with indistinguishable results (data not shown).

Construction of transgenic flies. The GAGA-581 cDNA was a generous gift of C. Benyajati. The GAGA-519 cDNA was obtained from K. Bhat. The GAGA-LacZ fusion was obtained by joining the LacZ coding sequence bearing a 3′-

untranslated region with a simian virus 40 poly(A) site at the *Sph*I site of GAGA-519. The GAGA-519 cDNA and the cDNA coding for the fusion protein were inserted into the *Xho*I-*Sal*I region of the hsp83CasPeR vector (5). The 3′ end of the GAGA-519 cDNA was then substituted for the 3′ end of a GAGA-581 cDNA. All plasmids were purified with Qiagen columns and injected into embryos from the w^1 stock. All the transgenes were marked by *mini-white* and were monitored by eye color.

Both *hsp83:GAGA-519* lines (#2 and #3) described here were on the second chromosome. One of the *hsp83:GAGA-581* lines (#12) was on the second, while the other (#13) was on the third. Two of the *hsp83:GAGA-LacZ* lines (#12 and #8) were on the third chromosome, and one (#6) was on the second. All lines exhibited comparable yellow-orange eye color with the exception of the *hsp83:GAGA-LacZ* line #6, which was pale yellow. All third-chromosome lines recombined readily with *Trl* alleles.

For simplicity, *hsp83:GAGA-519*-2 *hsp83:GAGA-519*-3; $Trl^{81.1}/Trl^{109.2}$ flies are referred to as 4X*hsp83:GAGA-519*; $Trl^{81.1}/Trl^{109.2}$, and *hsp83:GAGA-581*-12; *hsp83:GAGA-581*-13 Trl^{R67}/*hsp83:GAGA-581*-13 $Trl^{109.2}$ flies are referred to as 4X*hsp83:GAGA-581*; $Trl^{R67}/Trl^{109.2}$.

Genetic crosses. All crosses were performed at 22°C in an incubator. Flies were grown on standard cornmeal media.

In order to minimize the influences of the environment and genetic background, the viabilities of Trl^{13C}/Trl^{R67} flies that carried each of the transgenes were compared to those of their siblings that did not. These were generated by crossing w^1; Tg/+; Trl^{13C}/TM6b males or females to w^1; +/+; Trl^{R67}/TM6b males or females if the transgene was on the second chromosome and w^1; Tg Trl^{13C}/Trl^{13C} males to w^1; Trl^{R67}/TM6b females if it was on the third.

The *Trl*-null flies carrying four copies of *hsp83:GAGA-519* or *hsp83:GAGA-581* were obtained from a cross of parents heterozygous for null *Trl* alleles. To avoid second-site mutation effects, the flies were made trans-heterozygous for different null alleles.

Tests for female fertility and maternal effect. Single females were placed in laying blocks with three males each. The eggs produced by each female over a 24-h period were counted, and the numbers were averaged over 7 to 10 days. At least 4, but usually 10 females of each genotype were scored. The eggs produced were then left for an additional 48 h, and the hatch rate was calculated. All the tests were performed in an incubator at 22°C.

Statistics. χ^2 analysis was used when comparing numbers of progeny from one cross. When comparisons between crosses were made, two-tailed Fisher's exact test using 2×2 contingency tables was utilized. This test was used for comparing percentages of males with A6-to-A5 transformations as well.

Western blots. About 10 0- to 6-h-old embryos' worth of extract was loaded onto a sodium dodecyl sulfate–8% polyacrylamide gel. Western blotting was performed as described in reference 9. Blots were probed with a 1:1,000 dilution of rabbit α-GAGA-581 or a 1:500 dilution of rabbit α-GAGA-519 antibody (3). Crude sera were affinity purified as described in reference 3. For a loading control the bottom part of the filter was cut off and probed with a 1:15 dilution of anti-Snf monoclonal antibody 4G3 raised in mouse (8). Horseradish peroxidase-conjugated secondary antibodies at dilutions of 1:1,000 (α-mouse) and 1:10,000 (α-rabbit) were used. Signals were detected with the Lumi-Light (Boehringer Mannheim) chemiluminescence system.

Staining of polytene chromosomes. Larvae were grown at 22°C in plastic bottles on standard media regularly supplemented with water and yeast. When the first third-instar larvae began to appear, the bottles were switched to 29°C for 3 days to induce a high level of GAGA-LacZ expression. Salivary glands were dissected in phosphate-buffered saline (PBS) with 1% Tween 20 (Sigma) and fixed for 2 min in 1 drop of solution containing 50% acetic acid, 3.7% formaldehyde, and 1% Tween 20. Chromosomes were spread and stored in PBS with 0.2% Tween 20 and 1% bovine serum albumin (PBSTB) at 4°C. Affinity-purified rabbit α-GAGA-581 (3) was used at a dilution of 1:50. A mouse α-LacZ monoclonal antibody (obtained from the Developmental Studies Hybridoma Bank, University of Iowa) was used at a dilution of 1:50. Alexa-488- and Alexa-546-conjugated goat secondary antibodies (Molecular Probes) were used at a dilution of 1:1,000 in PBSTB with 5% normal goat serum. DNA was detected with Hoechst (0.5 μg/ml in water). Images were collected with the Zeiss LSM 510 confocal microscope.

Staining of embryos. Embryos were collected, fixed, and stained according to a standard protocol (4). Females of all genotypes were raised at 22°C. Embryos were collected at 22°C, except for embryos from 2X*hsp83:GAGA-LacZ* mothers. These were collected at 29°C. DNA was visualized using TOTO-1 at a dilution of 1:300. α-GAGA-519 and α-GAGA-581 were used at dilutions of 1:25 and 1:50, respectively. Staining was visualized by fluorescence using biotinylated goat α-rabbit or α-mouse antibodies and Alexa-488-conjugated streptavidin. Images were collected with the Zeiss LSM 510 confocal microscope.

RESULTS

GAGA transgenes express the GAGA-519 and GAGA-581 isoforms. To examine the in vivo functions of the two GAGA isoforms, we used an *hsp83* promoter to drive the expression of cDNAs encoding the GAGA-519 and GAGA-581 proteins (Fig. 1a). The *hsp83* promoter is active at room temperature in most fly tissues and cell types and can be upregulated by raising the flies at elevated temperatures (5). This promoter has been used previously to drive the expression of cDNA rescue constructs for ubiquitously expressed genes like *transformer*, *doublesex*, and *zw-5* (14, 34, 35). *Mini-white* transgenes containing the *hsp83:GAGA-519* and *hsp83:GAGA-581* expression constructs were introduced into flies and multiple independent insertion lines of each were obtained. Two lines for each transgene were then selected for further analysis. The appropriate GAGA isoform is expressed by each transgene. In the experiment shown in Fig. 1b, embryos were collected from *Trl*-null mutant females rescued by four copies of either the *hsp83:GAGA-519* or the *hsp83:GAGA-581* transgene and from wild type. Western blots were prepared from each sample and probed with antibodies specific to the GAGA-519 or GAGA-581 isoforms (see the legend to Fig. 1b). The maternally encoded GAGA-519 isoform (Fig. 1b, lane 1) is observed in 0- to 6-h-old wild-type embryos. This isoform is also found in embryos from 4X*hsp83:GAGA-519* mothers (lane 2), but it is not present in embryos from 4X*hsp83:GAGA-581* mothers (lane 3). Note that offspring from *Trl* mutant mothers that have four copies of the *hsp83:GAGA-519* transgene have higher levels of the GAGA-519 isoform than are found in wild-type embryos. Finally, the GAGA-581 isoform can be detected in embryos from 4X*hsp83:GAGA-581* mothers (see lanes 4 to 6) but not in embryos from wild-type or 4X*hsp83:GAGA-519* mothers. This was expected since little if any of this isoform is contributed maternally (3).

Both GAGA isoforms rescue the lethal effects of Trl mutant combinations. Flies homozygous for the hypomorphic Trl^{13C} mutation have reduced viability. This lethality is enhanced when Trl^{13C} is *trans* to one of the null *Trl* alleles such as Trl^{R67}, and only a few percent of the mutant flies survive to the adult stage (Fig. 2a). We found that a single copy of either the *hsp83:GAGA-519* or the *hsp83:GAGA-581* transgene can rescue the lethality of the Trl^{13C}/Trl^{R67}mutant combination (Fig. 2a). In a separate set of experiments with an *hsp70* promoter, Granok et al. (personal communication) also found that a cDNA encoding the GAGA-519 isoform can rescue the lethal effects of Trl^{13C}; however, the rescuing activity in their experiments was much less than that obtained with our *hsp83* transgenes, presumably because heat shock is required to activate the *hsp70* promoter. A comparison of the extent of rescue with the *hsp83* transgenes indicates that rescuing activity is influenced by chromosomal-position effects. Nevertheless, both of the *hsp83:GAGA-581* lines are more effective in restoring viability than either of the *hsp83:GAGA-519* lines.

We also tested whether the *hsp83:GAGA-519* or *hsp83:GAGA-581* transgenes can rescue *Trl*-null mutations. We found that the zygotic lethality of null allelic combinations could not be rescued by either a single or two copies of the transgenes. However, it was possible to rescue *Trl*-null mutant combinations using four copies of the transgenes (Fig. 2b).

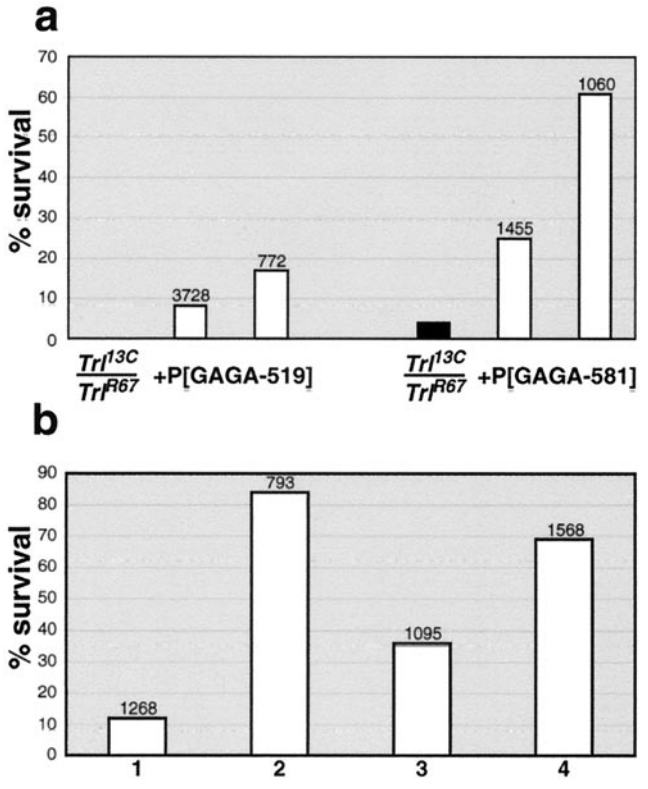


FIG. 2. GAGA transgenes rescue viability of hemizygous Trl^{13C} flies. Viability was calculated as the percentage of the expected numbers based on Mendelian ratios. Numbers above bars indicate the total number of flies scored in a cross. (a) Siblings with (white columns) and without (black columns) a transgene were compared. Different bars of the same genotype represent different lines of a transgene. The viability of Trl^{13C}/Trl^{R67} flies represents an average between two crosses involving different lines of the same transgene. All *P* values were smaller than 10^{-4}, obtained by the χ^2 test. (b) Viability of *Trl*-null flies rescued by four copies of *hsp83:GAGA-519*, four copies of *hsp83:GAGA-581*, or two copies of each. Genotypes of flies examined were 4X*hsp83:GAGA-519*; $Trl^{109.2}/Trl^{81.1}$ (Bar 1); 4X*hsp83:GAGA-581*; $Trl^{109.2}/Trl^{R67}$ (Bar 2); 2X*hsp83:GAGA-581*/2X*hsp83:GAGA-519*; $Trl^{109.2}/Trl^{81.1}$ (Bar 3); 2X*hsp83:GAGA-581*/2X*hsp83:GAGA-519*; $Trl^{109.2}/Trl^{R67}$ (Bar 4). The rescue was significant in each case, with all *P* values smaller than 10^{-4} by Fisher's exact test.

Although GAGA-581 appears to be more active than GAGA-519 in this situation as well, for technical reasons we were able to assay the activity of only four copies of each transgene using a different combination of *Trl*-null alleles. Hence, the relative activity of the *hsp83:GAGA-519* and *hsp83:GAGA-581* transgenes is best assessed by comparing the viability of equivalent *Trl* mutant combinations that have either four copies of one transgene or two copies of each transgene. As can be seen in Fig. 2b (compare the first and third bars), replacing two of the four copies of *hsp83:GAGA-519* with two copies of *hsp83:GAGA-581* improves the viability of this *Trl* mutant combination. Conversely, replacing two copies of *hsp83:GAGA-581* with two copies of *hsp83:GAGA-519* reduces the viability of the other *Trl* mutant combination (Fig. 2b, compare the second and fourth bars).

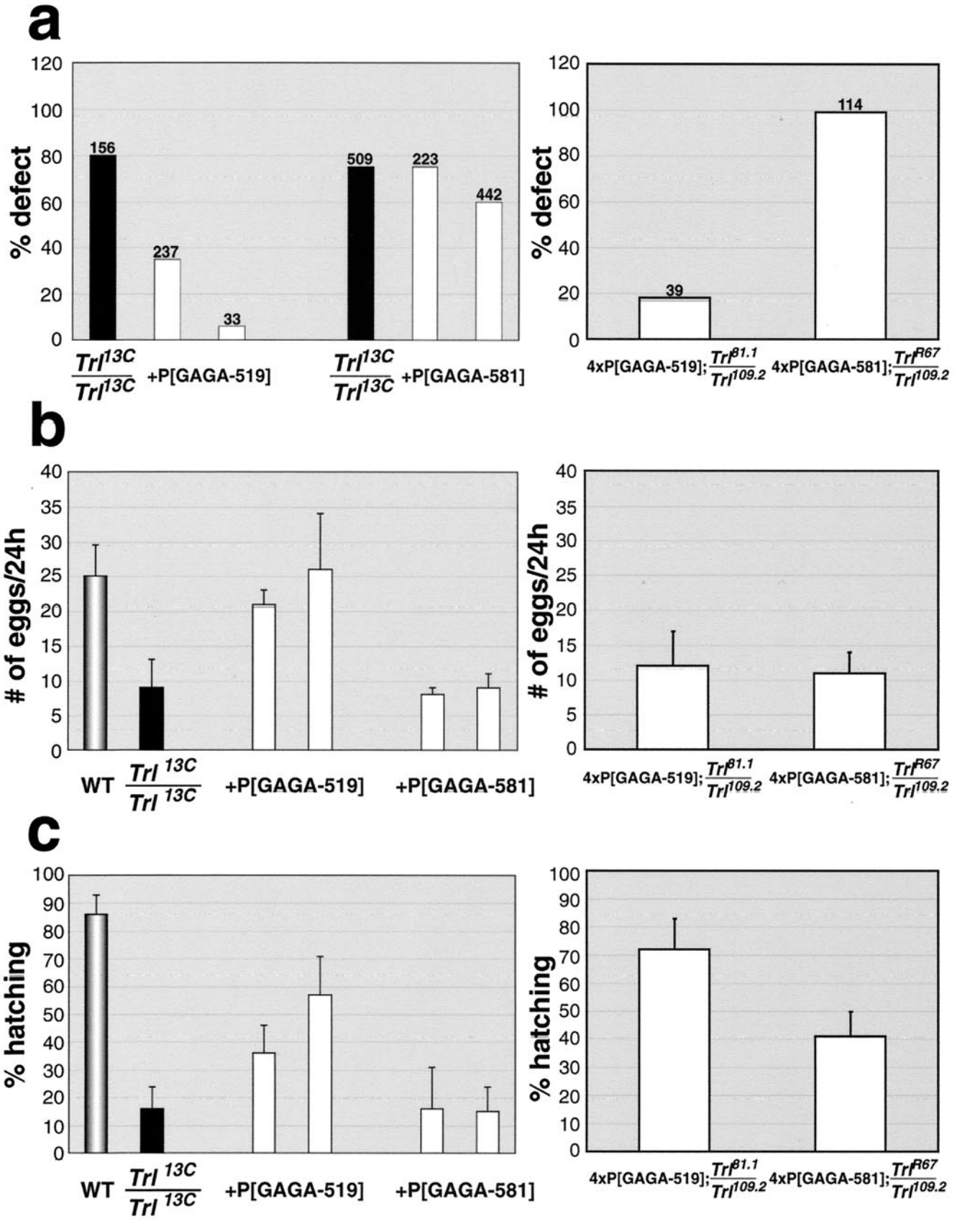
The GAGA-519 and GAGA-581 transgenes have overlapping but not equivalent activities. The results described in the previous section indicate that the *hsp83:GAGA-519* transgene is less effective in rescuing the zygotic lethality associated with loss of *Trl* function than is *hsp83:GAGA-581*. An obvious question is whether there are any other differences in the activities of the two transgenes. To address this question we compared the abilities of the two transgenes to rescue several other *Trl* mutant phenotypes. We used two different transgene-*Trl* mutant combinations for these experiments. In the first, we tested the rescuing activity of a single copy of the transgene in a Trl^{13C} homozygous mutant background. Since the Trl^{13C} allele is hypomorphic, it should be possible to detect some amelioration of the phenotype even if the transgene has only weak activity. In the second, we tested the rescuing activity of four copies of the transgene in *Trl*-null background. In this background, a much higher level of activity should be required to rescue the mutant phenotypes.

Abdominal transformation. About 80% of Trl^{13C} homozygous males show a transformation of the sixth abdominal segment towards the fifth, indicative of a loss of *Abd-B* activity (12). Since the introduction of a single copy of the *hsp83:GAGA-519* transgene into the Trl^{13C} mutant background suppresses this transformation, the GAGA-519 isoform can rescue this defect in *Abd-B* expression (Fig. 3a, left panel). By contrast, the *hsp83:GAGA-581* transgene has little rescuing activity. Similar results were obtained with four copies of the transgene in the *Trl*-null mutant background. All of the surviving *hsp83:GAGA-581 Trl* mutant males had the abdominal transformation (Fig. 3a, right panel). In contrast, less than 20% of the *hsp83:GAGA-519 Trl* mutant males had this transformation.

Egg-laying defects. Trl^{13C} females produce a reduced number of eggs compared to the wild type (Fig. 3b, left panel). This egg-laying defect can be rescued by the *hsp83:GAGA-519* transgene, but not by the *hsp83:GAGA-581* transgene. Interestingly, the two transgenes are equally effective in rescuing the egg-laying defects of *Trl*-null females (Fig. 3b, right panel).

Maternal-effect lethality. Fewer than 20% of the embryos produced by homozygous Trl^{13C} mothers survive to the larval stage, even when fertilized by wild-type males (Fig. 3c, left panel). The *hsp83:GAGA-519* transgene partially rescues this

FIG. 3. Rescue of *Trl* phenotypes by GAGA-519 and GAGA-581. (a) Rescue of abdominal transformations in males. Siblings with (white columns) and without (black columns) a transgene were compared. The number above each bar represents the total number of males examined. Values for Trl^{13C}-homozygous males shown represent averages between the crosses involving different lines of the same transgene. Rescue by GAGA-519 was highly significant, with all *P* values less than 10^{-8}, calculated by Fisher's exact test. (b) Rescue of female fertility. Error bars represent 95% confidence intervals. The numbers of eggs laid by single females were measured as described in Materials and Methods. In the left panel, the gray column corresponds to the wild-type females, the black column corresponds to the Trl^{13C}-homozygous females, and the white columns correspond to Trl^{13C}-homozygous females carrying the transgenes. (c) Rescue of maternal effect lethality. Error bars represent 95% confidence intervals. The hatch rate was determined after 48 h at 22°C, as described in Materials and Methods. The colors of the columns correspond to the same genotypes as for panel b.


a
% defect
120
100
80
60
40
20
0
156
237
33
509
223
442
Trl13C / Trl13C +P[GAGA-519]
Trl13C / Trl13C +P[GAGA-581]
39
114
4xP[GAGA-519]; Trlδ1.1 / Trl109.2
4xP[GAGA-581]; TrlR67 / Trl109.2
b
# of eggs/24h
40
35
30
25
20
15
10
5
0
WT Trl 13C / Trl 13C
+P[GAGA-519]
+P[GAGA-581]
c
% hatching
100
90
80
70
60
50
40
30
20
10
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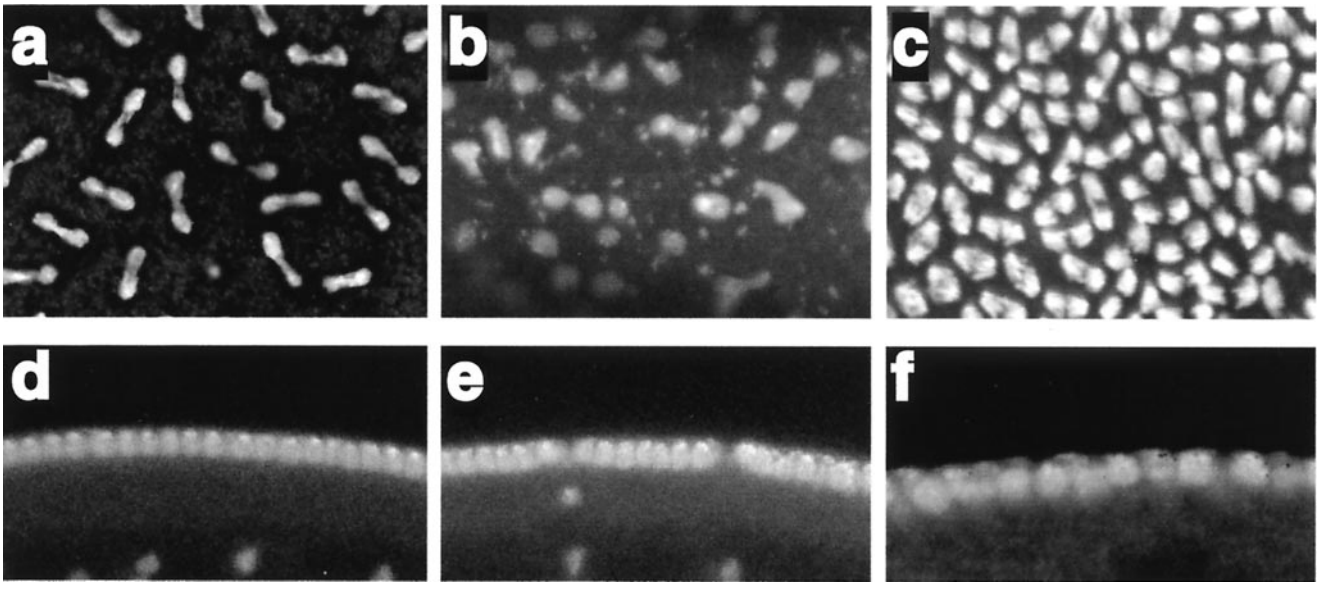

FIG. 4. GAGA-581 can rescue *Trl* nuclear defects. (a to c) Nuclei in mitosis. (d to f) Embryos at syncytial blastoderm stage. Nuclei were visualized with TOTO-1. (a and d) Embryos from w^1 mothers. (b and e) Embryos from Trl^{13C} mothers. (c and f) Embryos from 4X*hsp83:GAGA-581*; $Trl^{109.2}/Trl^{R67}$ mothers. All females were fertilized by w^1 males.

maternal-effect lethality, while *hsp83:GAGA-581* does not. In the *Trl*-null background, the *hsp83:GAGA-581* transgene is about half as active as *hsp83:GAGA-519* (Fig. 3c, right panel).

Nuclear division and transcription defects. A large proportion of the embryos from Trl^{13C} mothers have severe defects in nuclear division during the syncytial blastoderm stage. Consequently, the *hsp83:GAGA-581* transgene might be less able to rescue the maternal-effect lethality of *Trl* mutations because the GAGA-581 isoform cannot completely support nuclear division. To determine if this is the case, we examined embryos from wild-type, homozygous Trl^{13C} and 4X *hsp83:GAGA-581;* $Trl^{R67}/Trl^{109.2}$ mothers. As reported previously (4), the majority of embryos laid by homozygous Trl^{13C} mothers exhibited a variety of nuclear division and mitotic abnormalities (Fig. 4b), leading to the loss of nuclei from the surface of the embryo later on (Fig. 4e). In contrast, none of the embryos produced by 4X*hsp83:GAGA-581;* $Trl^{R67}/Trl^{109.2}$ mothers exhibited such defects (Fig. 4c), and the nuclear monolayer was intact at subsequent stages of embryogenesis (Fig. 4e). Nevertheless, fewer than half of these embryos hatched into first-instar larvae (Fig. 3c, right panel).

These findings argue that the GAGA-581 isoform is capable of supporting nuclear division in the early embryo. Hence, the failure to more completely rescue maternal-effect lethality is most likely due to an inability to function in some other GAGA-519-dependent process such as zygotic gene expression. With this possibility in mind, we examined *ftz* expression in embryos produced by Trl^{13C} mothers that either have or do not have a copy of the *hsp83:GAGA-581* transgene. We found that the GAGA-581 isoform rescues the Trl^{13C} defects in *ftz* expression (not shown). This would suggest that the misexpression of some other gene or some other *Trl*-dependent process is not fully rescued by the GAGA-581 protein.

Distribution of the two isoforms on mitotic chromosomes in early embryos. In wild-type embryos, high levels of the GAGA-519 protein localize to centromeric heterochromatin and can be visualized as brightly labeled dots in mitotic chromosomes (Fig. 5a). (Note that even greater amounts of centromeric GAGA-519 protein are present in embryos from 4X*hsp83:GAGA-519* mothers). In contrast, in embryos from Trl^{13C} homozygous mothers, where there are nuclear division defects, there is little or no GAGA protein in heterochromatin (4). Since the GAGA-581 protein is able to rescue the nuclear division defects, we expected that it should localize to centromeric heterochromatin like the GAGA-519 protein. Figure 5 shows that this is the case. The GAGA-581 protein was not detected in mitotic chromosomes of wild-type embryos but was localized to centromeric heterochromatin in embryos produced by mothers carrying four copies of the *hsp83:GAGA-581* transgene. Moreover, this localization was observed even in embryos produced by *Trl* mutant mothers.

GAGA-LacZ fusion protein retains substantial GAGA activity. Although the GAGA-519 and GAGA-581 proteins have distinct properties in vivo, they can, in many instances, function interchangeably. This led us to wonder whether the Q domains could be replaced by a heterologous protein. To test this possibility, we fused the coding sequences for the first 495 aa of GAGA-519 to sequences encoding the bacterial β-galactosidase protein. As illustrated in Fig. 6a, this partially eliminates the GAGA-519 Q domain and replaces it with β-galactosidase. The resulting fusion protein retains only 13 of the 30 glutamine residues found in the GAGA-519 Q domain. Three independent *hsp83:GAGA-LacZ* transgenic lines were selected for further study. Expression of the GAGA-LacZ fusion protein was verified by Western blotting (not shown) and by X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining of embryos (data not shown) and salivary glands (Fig. 7a).

We then examined the biological activity of the *hsp83:GAGA-LacZ* transgene. As shown in Fig. 6b, the zygotic lethality of the Trl^{13C}/Trl^{R67} mutant combination can be rescued

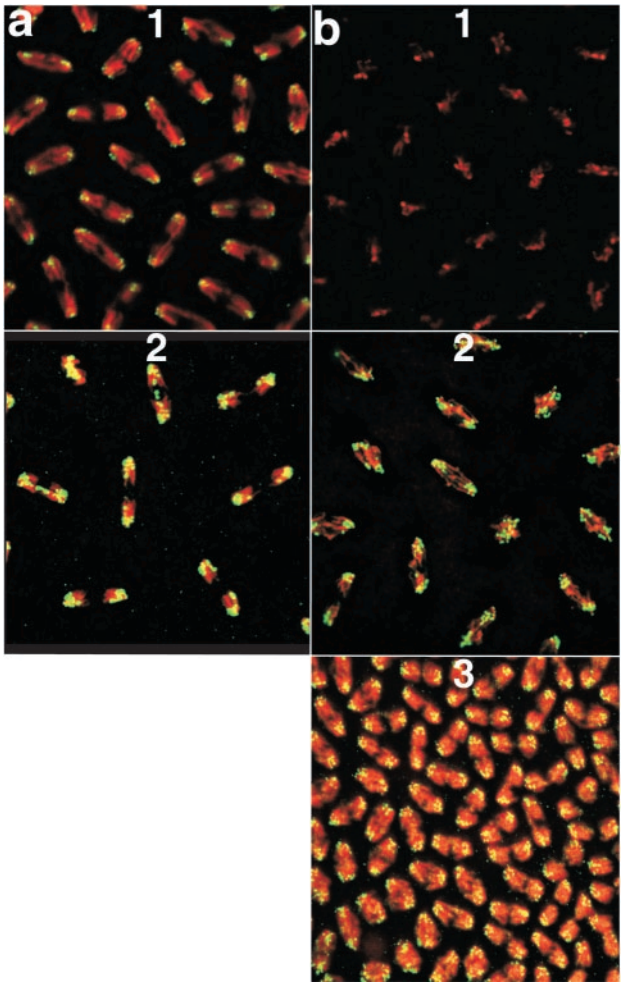

FIG. 5. Association of GAGA-519 and GAGA-581 with centric heterochromatin. DNA is in red, and protein is in green. Chromosomes are shown in anaphase, at the time they are being separated by microtubules. Centromeres are pointing outward, and telomeres are oriented inward. All embryos are at the syncytial blastoderm stage (stage 4 [36]) of embryogenesis but have undergone different numbers of nuclear divisions. Embryos were stained with the GAGA-519-specific antibody (a) or with the GAGA-581-specific antibody (b). DNA was visualized with TOTO-1. The genotypes of embryos were as follows: a1 and b1, wild type; a2, 4X*hsp83: GAGA-519*; b2, X*hsp83: GAGA-581*; b3, 4X*hsp83GAGA-581*; $Trl^{109.2}/Trl^{R67}$.

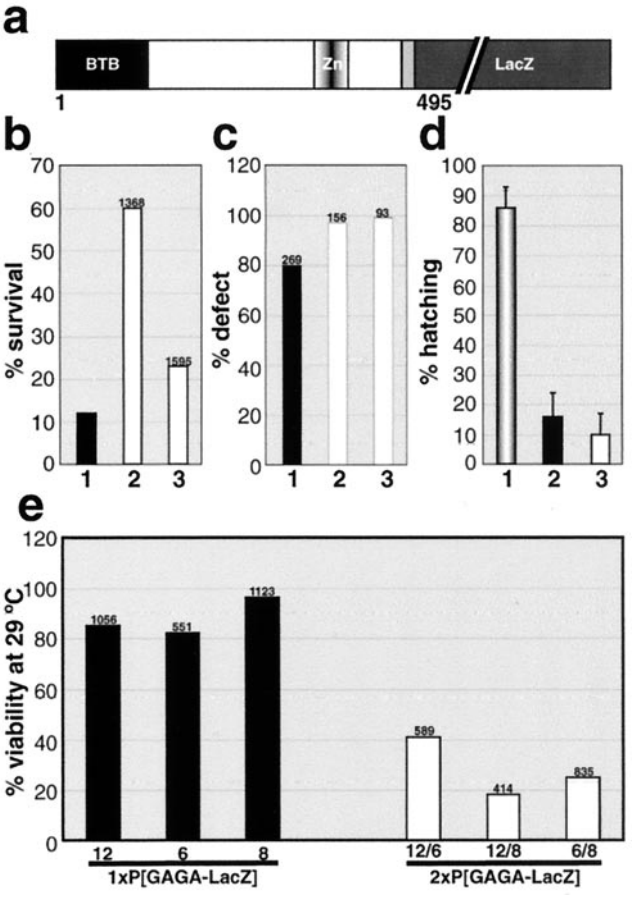

FIG. 6. Effects of *hsp83:GAGA-LacZ* on viability and *Trl* loss of function phenotypes. (a) A schematic representation of the LacZ fusion protein. Expression was driven with the *hsp83* promoter. (b) One copy of *hsp83:GAGA-LacZ* rescues viability of Trl^{13C}/Trl^{R67} flies. Genotypes represented are as follows: 1, Trl^{13C}/Trl^{R67}; 2, 1X*hsp83:GAGA-LacZ* #6; Trl^{13C}/Trl^{R67}; 3, 1X*hsp83:GAGA-LacZ* #8; Trl^{13C}/Trl^{R67}. Siblings with and without the transgene were compared. The viability of Trl^{13C}/Trl^{R67} represents an average between two crosses involving different lines of the transgene. All *P* values were smaller than 10^{-4}, obtained by the χ^2 test. (c) Rescue of abdominal transformations in males. The genotypes of males examined were as follows: bar 1, Trl^{13C}/Trl^{13C}; bar 2, 1X*hsp83: GAGA-LacZ* #6; Trl^{13C}/Trl^{13C}; bar 3, 1X*hsp83: GAGA-LacZ* #8; Trl^{13C}/Trl^{13C}. Siblings with and without the transgene were compared. The number above each bar represents the total number of males examined. Values for Trl^{13C}-homozygous males shown represent averages between two crosses involving different lines of the transgene. (d) Rescue of maternal-effect lethality. The genotypes of females shown are as follows: bar 1, w^1; bar 2, Trl^{13C}/Trl^{13C}; bar 3, 1X*hsp83:GAGA-LacZ* #6; Trl^{13C}/Trl^{13C}. Error bars represent 95% confidence intervals. The hatch rate was determined after 48 h at 22°C, as described in Materials and Methods. (e) Lethal effects of the *hsp83: GAGA-LacZ* transgene in one copy (black bars) and two copies (white bars). The number above each bar indicates the total number of flies scored in a cross. The viability of flies with one copy of the transgene was compared to that of w^1 siblings. The viability of flies carrying two copies of the transgene was compared to that of heterozygous siblings. Effects in two copies were assayed in trans-heterozygotes to avoid site of insertion effects. All *P* values for two-copy lethality were less than 10^{-11}, calculated using the χ^2 test.

by a single copy of the *hsp83:GAGA-LacZ* transgene. In fact, one of the transgenic lines appears to rescue zygotic lethality to almost the same extent as the *hsp83:GAGA-581* transgene. On the other hand, the *hsp83:GAGA-LacZ* transgene does not rescue either the A6-to-A5 abdominal transformation or the maternal-effect lethality of Trl^{13C}/Trl^{R67} flies (Fig. 6c and d). The fusion protein does, however, localize to centromeric heterochromatin in the mitotic chromosomes of early embryos (Fig. 7d). The activity of *hsp83:GAGA-LacZ* transgenes could not be assayed in *Trl*-null background because we were unable to obtain flies carrying more than two copies of the transgene even in a wild-type background (see below).

GAGA-LacZ binds sites not associated with wild-type GAGA proteins. Although the *hsp83:GAGA-LacZ* transgene is able to rescue some of the phenotypic effects of *Trl* mutations, the fusion also has some unusual activities. While a single copy of the *hsp83:GAGA-LacZ* transgene has little effect on viability, two copies of the transgene significantly reduced viability at higher temperatures. As shown in Fig. 6e, the viability of all

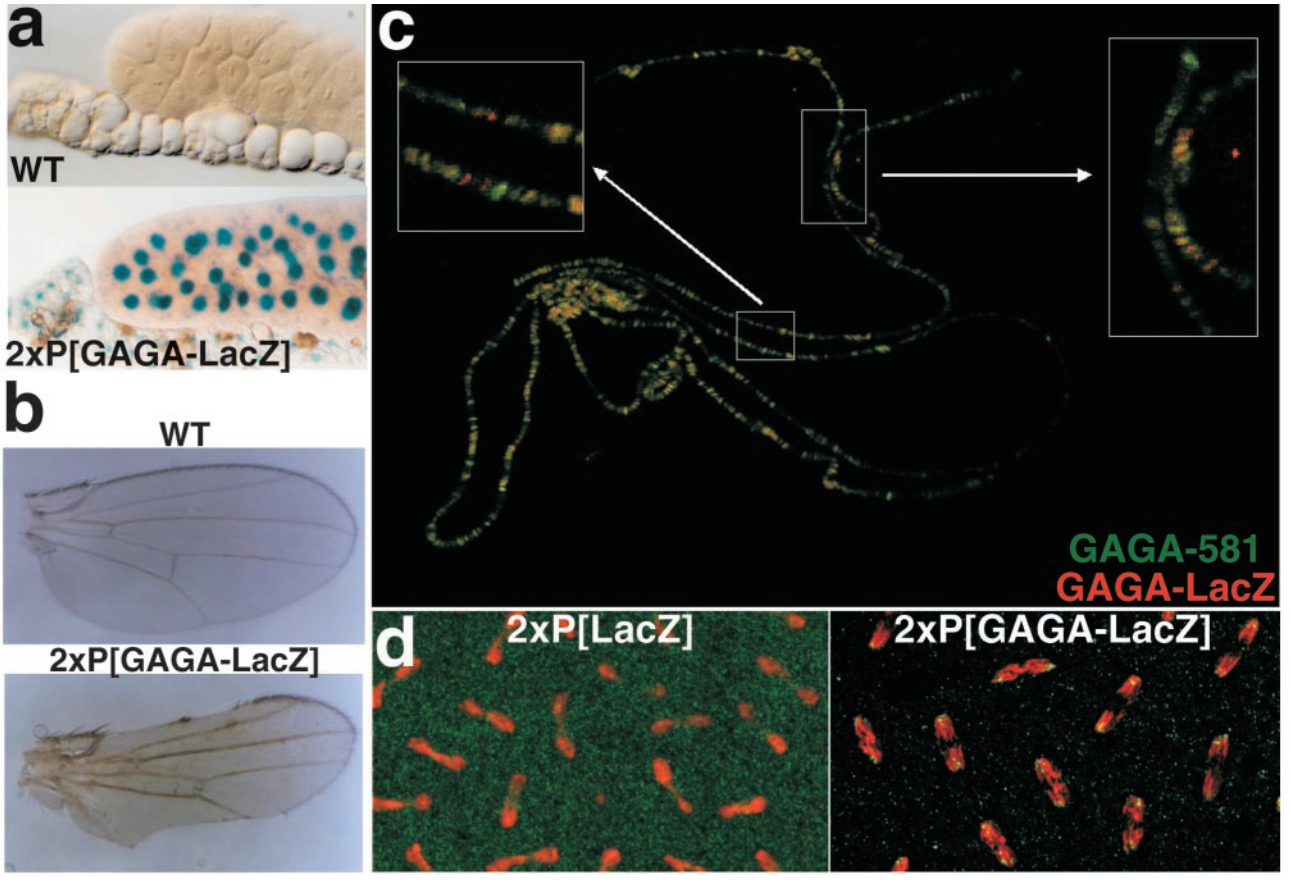

FIG. 7. Localization of the *hsp83:GAGA-LacZ* transgene. (a) Salivary glands from wild-type (WT) and 2X*hsp83:GAGA-LacZ* larvae stained with X-Gal. Line #8 of *hsp83:GAGA-LacZ* was used. Other lines showed similar staining patterns and intensities (data not shown). (b) Wing defects associated with GAGA-LacZ. Flies were raised at 29°C, and wings were dissected off and mounted in Hoyer's mountant. (c) Imperfect colocalization of GAGA-581 (green) and GAGA-LacZ (red). The overlap is yellow. Polytene chromosomes were double stained using antibodies against GAGA-581 and LacZ, as described in Materials and Methods. (d) Association of GAGA-LacZ with centric heterochromatin. DNA is in red, and protein is in green. As in Fig. 4, chromosomes are shown in anaphase, at the time they are being separated by microtubules. Centromeres are pointing outward, and telomeres are oriented inward. Embryos were stained with an antibody against LacZ; DNA was visualized with TOTO-1 (see Materials and Methods).

three pairwise combinations of *hsp83:GAGA-LacZ* inserts was less than 50% at 29°C. Similar lethal effects are observed when there are two copies of the same insert. The 2X*hsp83:GAGA-LacZ* flies that survive at 29°C exhibit phenotypes, such as the loss of the wing margin (Fig. 7b), not normally associated with *Trl* loss of function. Since neither of these phenotypic effects becomes more severe when the transgenic flies are heterozygous for the Trl^{R67} mutation, it would appear that the *hsp83:GAGA-LacZ* transgene mimics a neomorphic allele of *Trl*.

One plausible explanation for the neomorphic activity of the GAGA-LacZ protein is that it interacts with novel sites in the genome, interfering with the regulation of genes located near these ectopic sites. To test this possibility, we double labeled polytene chromosomes using antibodies against β-galactosidase and the GAGA-581 protein. In most, but not all cases, we were able to detect the GAGA-LacZ fusion at sites that have the GAGA-581 protein (Fig. 7c). Since the signal produced by the β-galactosidase antibody is significantly fainter than that from the GAGA-581 antibody, it is possible that the few sites for the endogenous GAGA protein that are not labeled with the anti-LacZ antibody may actually have the GAGA-LacZ protein. On the other hand, we found several sites that have the GAGA-LacZ fusion but not the GAGA-581 protein. Although this result indicates that the in vivo specificity of the GAGA-LacZ fusion differs from that of the normal GAGA protein, we cannot rule out the possibility that some other mechanism is responsible for the neomorphic activity of the fusion protein.

DISCUSSION

The *Drosophila* GAGA factor functions in the establishment and/or maintenance of nucleosome-free regions of chromatin and has been implicated in processes as diverse as chromosome condensation and segregation, gene regulation, *Polycomb-group* silencing, and insulator activity (4, 7, 18, 21–23, 32). There are two major GAGA isoforms in *D. melanogaster*, GAGA-519 and GAGA-581, which have completely different C-terminal Q domains. In spite of this difference, the two isoforms behave identically in in vitro DNA binding experiments and in tissue culture transient-transfection assays (3). The two proteins also appear to colocalize completely on poly-

tene chromosomes (3). These findings have raised the possibility that these two GAGA isoforms may be functionally equivalent.

In the studies reported here, we have tested this hypothesis by examining how the isoforms function in vivo. We find that they have overlapping but not identical activities and, consequently, are not interchangeable. Although both isoforms can rescue the zygotic lethality of *Trl* mutations, the GAGA-581 isoform is more effective. On the other hand, with respect to most other known *Trl* phenotypes, the GAGA-519 protein seems to be more active. Thus, the homeotic transformation of segment A5 to A6, which is likely due to *Abd-B* misexpression, can be rescued by the GAGA-519 protein, but not by the GAGA-581 protein. Similarly, both female sterility and maternal-effect lethality are rescued more effectively by the GAGA-519 protein. At least two factors account for the maternal-effect lethality of *Trl* mutations (4). The initial defect in the offspring of Trl^{13C} mothers is the disruption of the nuclear division cycles at the syncytial blastoderm stage. Subsequently, genes that have GAGA binding sites in their regulatory regions are not properly expressed. Interestingly, the GAGA-581 protein, like the GAGA-519 protein, appears to fully rescue the nuclear division defects. Moreover, as is observed for the GAGA-519 protein, it preferentially localizes to centromeric heterochromatin in blastoderm nuclei. Since little if any GAGA-581 is present at this stage in wild-type embryos, we cannot say whether it normally functions at centromeres or is involved in chromosome mechanics at other stages of development. However, our results do suggest that centromere binding and GAGA function during mitosis do not depend upon the exact sequence and length of the Q domain. Thus, the GAGA-581 protein must be unable to substitute for the GAGA-519 protein in some other vital process during embryogenesis. At this point the most likely process is transcription. However, since both isoforms are able to rescue defects in *ftz* transcription, we presume that the GAGA-519 protein would have to have some other gene-specific function, perhaps equivalent to its role in *Abd-B* expression.

We can envision two possible explanations for the differences in the biological activities of the GAGA-519 and GAGA-581 proteins. Since the Q domains have distinct sequences, they may not be able to mediate the same spectrum of protein-protein interactions. Although this seems very likely to be the case, it is also possible that the two proteins cannot bind or do not bind with equal efficiency to all of the same target sites. The idea that the Q domains influence or modulate site selection in vivo is supported by the finding that the GAGA-LacZ fusion binds to novel sites (and may not bind to all of the normal GAGA sites) in polytene chromosomes. While this idea is contrary to the conclusions from previous studies (3), it seems possible that the in vitro binding assays were not sensitive enough to detect subtle differences in sequence preference that could be important in vivo, especially in an environment that has many other proteins that could potentially compete for the same sequences. Similarly, although GAGA-581 and GAGA-519 appear to colocalize on polytene chromosomes (3), the resolution of this experiment is not sufficient to conclude that the two proteins are binding to precisely the same sequences rather than nearby or adjacent sequences, or that they are binding with the same avidity. Since the Q domain of GAGA-519 appears to influence the topology of DNA upon GAGA factor binding in vitro (37), it is not unreasonable to suppose that this domain could influence the affinity of the protein for some sites.

It is somewhat surprising that the GAGA-LacZ fusion is able to rescue the zygotic lethality of *Trl* mutations almost as well as the GAGA-581 isoform does. It could be argued that the GAGA-519 Q domain sequence included in the GAGA-LacZ fusion is responsible for its rescuing activity. However, the activity profile of the GAGA-LacZ fusion more closely resembles that of GAGA-581 than that of GAGA-519. An alternative possibility is suggested by the finding that the GAGA-519 Q domain promotes the formation of tetrameric protein complexes (37). Not only does β-galactosidase form tetramers but also tetramerization is required for enzymatic activity (13). Since the GAGA-LacZ fusion protein has enzymatic activity (see Fig. 7a), it would appear that it assembles into tetrameric complexes. Hence, a quite plausible hypothesis is that β-galactosidase is able to partially substitute for the Q domain because of its ability to tetramerize. Conversely, the fact that the GAGA-LacZ fusion can rescue zygotic lethality would imply that (the postulated) protein-protein interactions mediated specifically by the fully intact Q domain are not critical for some of the general GAGA functions in the zygote such as chromatin remodeling and the formation of nucleosome free regions of chromatin. On the other hand, since the GAGA-LacZ protein lacks some of the activities of the GAGA-519 protein, it would appear that the C-terminal sequences deleted in the fusion are important for the special functions of this isoform. This is, of course, consistent with the finding that the activities of the GAGA-519 and GAGA-581 isoforms are not identical.

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