Article

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Abstract

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The GAGA factor is required in the early *Drosophila* embryo not only for transcriptional regulation but also for nuclear division

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SUMMARY

The GAGA protein of *Drosophila* was first identified as a stimulatory factor in in vitro transcription assays using the *engrailed* and *Ultrabithorax* promoters. Subsequent studies have suggested that the GAGA factor promotes transcription by blocking the repressive effects of histones; moreover, it has been shown to function in chromatin remodeling, acting together with other factors in the formation of nuclease hypersensitive sites in vitro. The GAGA factor is encoded by the *Trithorax-like* locus and in the studies reported here we have used the maternal effect allele Trl^{13C} to examine the functions of the protein during embryogenesis. We find that GAGA is required for the proper expression of a variety of developmental loci that contain GAGA binding sites in their upstream regulatory regions. The observed disruptions in gene expression are consistent with those expected for a factor involved in chromatin remodeling. In addition to facilitating gene expression, the GAGA factor appears to have a more global role in chromosome structure and function. This is suggested by the spectrum of nuclear cleavage cycle defects observed in Trl^{13C} embryos. These defects include asynchrony in the cleavage cycles, failure in chromosome condensation, abnormal chromosome segregation and chromosome fragmentation. These defects are likely to be related to the association of the GAGA protein with heterochromatic satellite sequences which is observed throughout the cell cycle.

Key words: GAGA, *Trithorax-like*, chromatin, nuclear division, heterochromatin, *Drosophila*

INTRODUCTION

In order to fit the very large genomes of higher eukaryotes inside the nucleus, the chromosomal DNA must be packaged into a complex nucleoprotein structure, chromatin. The primary subunits of chromatin are the nucleosome core particles which are distributed along the DNA like beads-on-a-string. This beads-on-a-string array represents only the first level of compaction and additional levels are required to fit the chromosomal DNA inside the nucleus. While this nucleoprotein complex condenses eukaryotic chromosomes many fold over B-form DNA, the DNA must still be used as a substrate for replication, recombination and transcription. That chromatin poses a significant hindrance to the proteins involved in these processes has been amply demonstrated (Grunstein, 1990; Felsenfeld, 1992; Kornberg and Lorch, 1992). One of major impediments is that of accessibility. This problem is generally solved by the placement of key target sequences in regions that are nucleosome free. Such nucleosome free segments can be recognized by their nuclease hypersensitivity (Wu, 1980). Thus, the control elements of genes transcribed by polymerases I, II, and III are typically located in short nuclease hypersensitive DNA segments which are devoid of nucleosomes (Elgin, 1988). Similarly, the in vivo sites of action for enzymes that are involved in other aspects of DNA metabolism, such as topoisomerase II, are generally restricted to sequences that are readily accessible to nucleases in chromatin digests (Reitman and Felsenfeld, 1990; Udvardy and Schedl, 1991; Kas and Laemmli, 1992; Kroeger and Rowe, 1992).

The mechanisms responsible for generating and maintaining nucleosome free regions of chromatin are not yet well understood. One protein that appears to function in this process is the *Drosophila* GAGA factor. It was first identified as a sequence specific DNA binding protein which could stimulate the transcriptional activity of the *engrailed* (*en*) and *Ultrabithorax* (*Ubx*) promoters in vitro (Soeller et al., 1988; Biggin and Tjian, 1988). Since then binding sites for the GAGA protein have been identified in the promoters of many other *Drosophila* genes, including *fushi tarazu* (*ftz*) (Topol et al., 199l), the *hsp26* (Glaser et al., 1990; Lu et al., 1993) and *hsp70* heat shock genes (Lee et al., 1992) and the histone H3-H4 promoters (Gilmour et al., 1989). Though GAGA was initially thought to be a standard transcription factor, several lines of

evidence have suggested that it may have a rather different function. First, GAGA does not appear to stimulate expression by interacting with the transcriptional machinery; instead it appears to act as an 'anti-repressor', preventing inhibitory factors such as histone H1 from blocking in vitro transcription (Croston et al., 199l). Second, studies on the chromatin structure of the *hsp26* promoter in vivo indicate that GAGA sequences are required for the formation or maintenance of nuclease hypersensitive regions (Glaser et al., 1990; Lu et al, 1993). Third, in in vitro chromatin assembly experiments, the GAGA factor promotes the formation of a nuclease hypersensitive region over the *hsp70* promoter (Tsukiyama et al., 1994).

To better understand how GAGA functions in vivo we have taken a genetic approach. Our initial entry was provided by a hypomorphic mutation, Trl^{13C} which has a P-element insertion in the first intron of the GAGA gene (Farkas et al., 1994). Trl^{13C} was identified because it dominantly enhances the weak segmental transformations observed in *Ubx* heterozygotes. This genetic interaction is a characteristic property of mutations in a class of loci known as the *trithorax*-group, and hence this locus was called *Trithorax-like*. Two presumed null alleles, Trl^{R67} and Trl^{R85}, which have small deletions in the coding sequence were generated by imprecise excision of the Trl^{13C} P-element. Since the promoters of many developmental loci have GAGA binding sites (see above), it was expected that animals homozygous for these mutations would die during embryogenesis and show extensive patterning defects. However, the lethal phase is not during embryogenesis. Rather it is later, during the larval stages. Moreover, the mutant animals exhibit no readily apparent patterning defects. A plausible explanation for this finding is that there is a substantial maternal deposition of *Trl* gene products which enables the mutant animals to develop to the larval stages in the absence of zygotic *Trl* gene activity. Indeed, GAGA protein can be detected in western blots of homozygous Trl^{R85} larvae (Farkas, unpublished data).

These observations suggested that we might best understand the functions of *Trl* in chromosome structure and gene regulation by reducing or eliminating this maternal contribution. The original hypomorphic Trl^{13C} allele is of interest in this respect. While homozygous Trl^{13C} animals have substantially reduced viability, a few survive to the adult stage. Significantly, Trl^{13C} females are sterile, laying a small a number of eggs which fail to develop properly. We have determined the likely basis for this female sterility and have examined the developmental defects of embryos produced by mutant mothers.

MATERIALS AND METHODS

Fly strains and genetics

Though the Trl^{13C} allele substantially reduces viability, a few homozygous animals survive to adulthood. While mutant females lay eggs, the number is quite modest (20-30 eggs per fly) compared to wild type (30-50 eggs per day). The defects in egg production appear to be due to abnormalities in the somatic tissues of the gonad and to the functioning of the stem cells (unpublished observations). Trl^{13C} was balanced with *TM3* or *TM6b*. The wild type was Oregon R. Other fly strains include two wild-type revertants of Trl^{13C}, *R4* and *R6*, which were generated by excision of the P-transposon, and a second independent *Trl* allele, Trl^{62} (Farkas et al., 1994). Like Trl^{13C}, Trl^{62} is also due to a P-element insertion; however it is more severe than Trl^{13C}, and animals homozygous for Trl^{62} die during the third instar larval stage. Although *trans*heterozygotes for Trl^{13C}/Trl^{62} can be recovered they are even less viable than Trl^{13C} homozygotes. Like the homozygotes the *trans*heterozygotes are female sterile (Farkas et al., 1994).

Northern analysis, western blotting and whole mounts

Samples for northern analysis were obtained from 5- to 10-day old Trl^{13C} homozygous ovaries or Trl^{13C} homozygous females and males. As a control, samples were also prepared from 5- to 10-day old wild-type ovaries and adults. RNA and protein samples were prepared according to the method of Chomozynski (1993). Developmental northern analysis was from the wild type. In northern analysis of samples from homozygous Trl^{13C} ovaries or adults and wild-type ovaries and adults, 15 μg of total RNA were used. For developmental northern analysis, poly(A)$^+$ RNA were used. Poly(A)$^+$ selection and northern analysis were performed as described by Sambrook et al. (1989). For western blotting analysis, samples were separated on a 7% gel in an SDS-PAGE system. The immunodetection was performed using ECL western blotting with slight modifications. The actin staining was done by cutting out and staining the strip containing the approximately 45×10^3 M_r actin isoform. For GAGA detection, rabbit antiserum against GAGA was used (a gift from C. Wu). Whole-mount in situ hybridization was performed essentially as described by Lantz et al. (1994). Immunostaining and confocal analysis of embryos and ovaries were performed essentially as described previously (Bhat and Schedl., 1994; Bhat et al., 1995). Antibodies against following proteins were used: GAGA (1:500 dilution; 25 minutes fixation), histone 2A (1:200 dilution; 25 min fixation), core histones (1:50 dilution; 25 min fixation), En (1:4 dilution; 25 minutes fixation), Ftz (1:200 dilution; 25 minutes fixation). Antibodies against the GAGA protein have been previously characterized by Tsukiyama et al. (1994) and Raff et al. (1994).

RESULTS

Trl^{13C} has a maternal effect lethal phenotype

A small percentage of homozygous Trl^{13C} females survive to the adult stage and produce at least some eggs. Since these eggs fail to develop even when fertilized by a wild-type male (see Table 1) we presume that the P-element insertion in Trl^{13C} results in a maternal effect lethality (MEL). As indicated in

Table 1. The maternal effect lethality in Trl^{13C}

		Developmental arrest (%)		
Genotype	Stage picked	Syncytial	Stage 11-15	Hatching
$\frac{Trl}{Trl}$ F × Wt M	stage 1	85	7.5	7.5
$\frac{Trl}{Trl}$ F × $\frac{Trl}{TM3}$ M	stage 1	61	17	22
$\frac{Trl}{Trl}$ F × $\frac{Trl}{Trl}$ M	stage 1	79	13	8

Homozygous Trl^{13C} females were mated to wild-type males, $Trl^{13C}/TM3$ males or to *Trl/Trl* males. Embryos were collected (at room temperature) and examined under halocarbon oil to assess the extent of development. Embryos were staged according to Wieschaus and Nüsslein-Volhard (1986). In general, most of the embryos from Trl^{13C} females arrest prior to the formation of the cellular blastoderm: class I. The remaining either develop upto about stage 11-15 (class II), or develop more or less normally to hatching (class III). It should be noted that the frequency of embryos falling into each class can vary between different egg collections. As a control, embryos from wild type or $Trl^{13C}/TM6b$ were also scored. About 95% of the embryos produced by a wild-type strain would hatch.

Table 1, there is some variability in the MEL phenotypes and the embryos can be roughly grouped into three classes based on their developmental defects. A majority are in class I. Embryos in this class arrest either before or during the syncytial blastoderm stage, prior to cellularization. The remaining embryos cellularize and commence with gastrulation. Of these, roughly half begin showing readily apparent developmental abnormalities during germband extension, between stages 11 and 15 (class II). In the other half, gastrulation is more or less normal; however, the embryos subsequently display a variety of segmentation defects and die by the first instar larval stage (class III). Within these two classes there is a continuum in the severity of the defects, with some embryos showing more extensive developmental abnormalities than others. For reasons that are not fully understood the relative number of embryos falling into each of these groups typically varies from one egg collection to another.

Two observations indicate that the maternal effect lethality of Trl^{13C} is likely due to the lesion in the GAGA gene. First, the maternal effect lethality (as well as all of the other phenotypes) is reverted by excision of the Trl^{13C} P-element transposon (Farkas et al., 1994; Gausz and Gyurkovics, unpublished data). Second, females *trans*heterozygous for Trl^{13C} and an independent *Trl* allele (Trl^{62}) are also female sterile and produce eggs exhibiting a similar range of phenotypes.

GAGA protein expression in wild-type and Trl^{13C} ovaries

The above findings suggests that the Trl^{13C} mutation interferes with the proper deposition of maternal GAGA gene products. To determine if this is the case, we first examined the distribution of GAGA protein in wild-type and Trl^{13C} mutant females (Fig. 1). In both wild-type and mutant adults, there appears to be little GAGA protein in the carcasses. By contrast, two major and three minor GAGA protein species are observed in ovaries. While Trl^{13C} ovaries typically have lower levels of GAGA protein than wild type (see Fig. 1), this reduction is rather modest and would not, in itself, be expected to be a major contributing factor to the Trl^{13C} maternal effect lethality.

This conclusion is supported by confocal analysis of the GAGA protein distribution in wild-type and Trl^{13c} mutant ovaries. The distribution of GAGA protein at different stages of oogenesis is shown for wild-type and Trl^{13c} ovaries in Fig. 2. To better discern how GAGA is distributed in different ovarian cell types we counterstained with antibodies against Orb (which localizes in the oocyte; Lantz et al., 1994). In region 1 of the germarium, GAGA is present in the nuclei of stem cells, cystoblasts and the 2-16 cell cysts (Fig. 2A). As the 16-cell cysts proceed through regions 2 and 3 of the germarium, GAGA can be detected in nurse cells and the oocyte (compare GAGA and Orb protein localization in Fig. 2A). However, soon after the egg chambers exit the germarium, around stage 3-4, GAGA protein in the oocyte nucleus begins to disappear (Fig. 2B), and by stage 7-8 (Fig. 2C) only a faint spot is evident. By stage 10 (Fig. 2D) GAGA can no longer be detected in the oocyte nucleus. It is also important to note that no GAGA protein is found in the ooplasm.

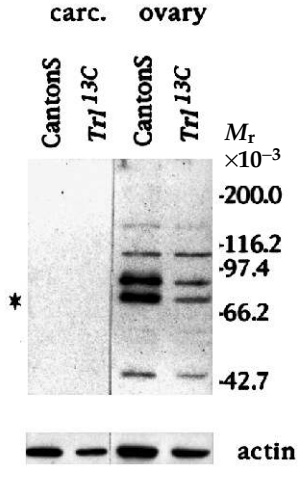

Fig. 1. GAGA protein is moderately reduced in Trl^{13C} ovaries. (Upper panel) western blots of protein (approx.6 μg) from ovaries and carcass of Trl^{13C} and wild-type females were probed with GAGA antibody (Tsukiyama et al, 1994). (Lower panel) As a control for loading, a strip from the same blot was cut from around the 45×10^3 M_r region and probed with actin antibody. Two major bands of approx. 67 (*) and approx. 80×10^3 M_r, and at least three minor bands are detected by the GAGA antibody. While the protein profile in Trl^{13C} ovaries is similar to wild type, there is a modest reduction in the level.

The chromosomal DNA of the nurse cells undergoes multiple rounds of endomitotic replication during the pre-vitellogenic stages of oogenesis (stages 1-7). Although GAGA persists in nurse cell nuclei during this period, the amount of protein appears to decrease. This is illustrated by the weaker, mottled staining observed the older previtellogenic chambers (Fig. 2B). This reduction is even more evident by early vitellogenesis (Fig. 2C). Moreover, the residual GAGA protein in the early vitellogenic nurse cell nuclei does not even appear to be associated with chromosomal DNA; instead it is localized in weakly staining foci distributed along the nuclear envelop (Fig. 2C). By mid-vitellogenesis, GAGA disappears from the nurse cells. In contrast to the germ cells, GAGA can be readily detected in the follicle cells of vitellogenic egg chambers (Fig. 2D), and is present at very high levels in these cells even in old egg chambers (data not shown).

The overall pattern of GAGA protein accumulation in germ cells of Trl^{13C} ovaries generally resembles that of wild type. As illustrated in Fig 2E, high levels of protein are found in germ cell nuclei in the germarium (open arrow), and in early egg chambers. Like wild type, the level of GAGA protein in older egg chambers (see Fig. 2E,F) is reduced in nurse cell nuclei and is absent in the oocyte.

Two conclusions can be drawn from these findings. First, the maternal stockpile of *Trl* gene products cannot be protein. Second, the level and distribution of GAGA protein in Trl^{13C} mutant egg chambers is not dramatically different from wild type. Thus a defect in GAGA protein production during oogenesis is unlikely to account for the maternal effect embryonic lethal phenotype of Trl^{13C} females.

The maternal effect lethality of Trl^{13C} is due to a defect in the expression of maternal *Trl* RNA

We next examined the expression of *Trl* mRNA in ovaries by whole mount in situ hybridization. The pattern of *Trl* RNA accumulation is different from that of GAGA protein. At early stages of oogenesis, when the level of GAGA protein is highest, the amount of *Trl* RNA is relatively low (Fig. 3A). After the onset of vitellogenesis, when there is only a little GAGA protein remaining in the egg chamber, there is a substantial increase in *Trl* RNA accumulation, and a strong hybridization signal is observed in nurse cells (Fig. 3A). While *Trl* RNA is also found in the oocyte during early and mid-vitellogenesis, the level of RNA is lower than in the nurse cells and appears to be largely restricted to the anterior. High levels of

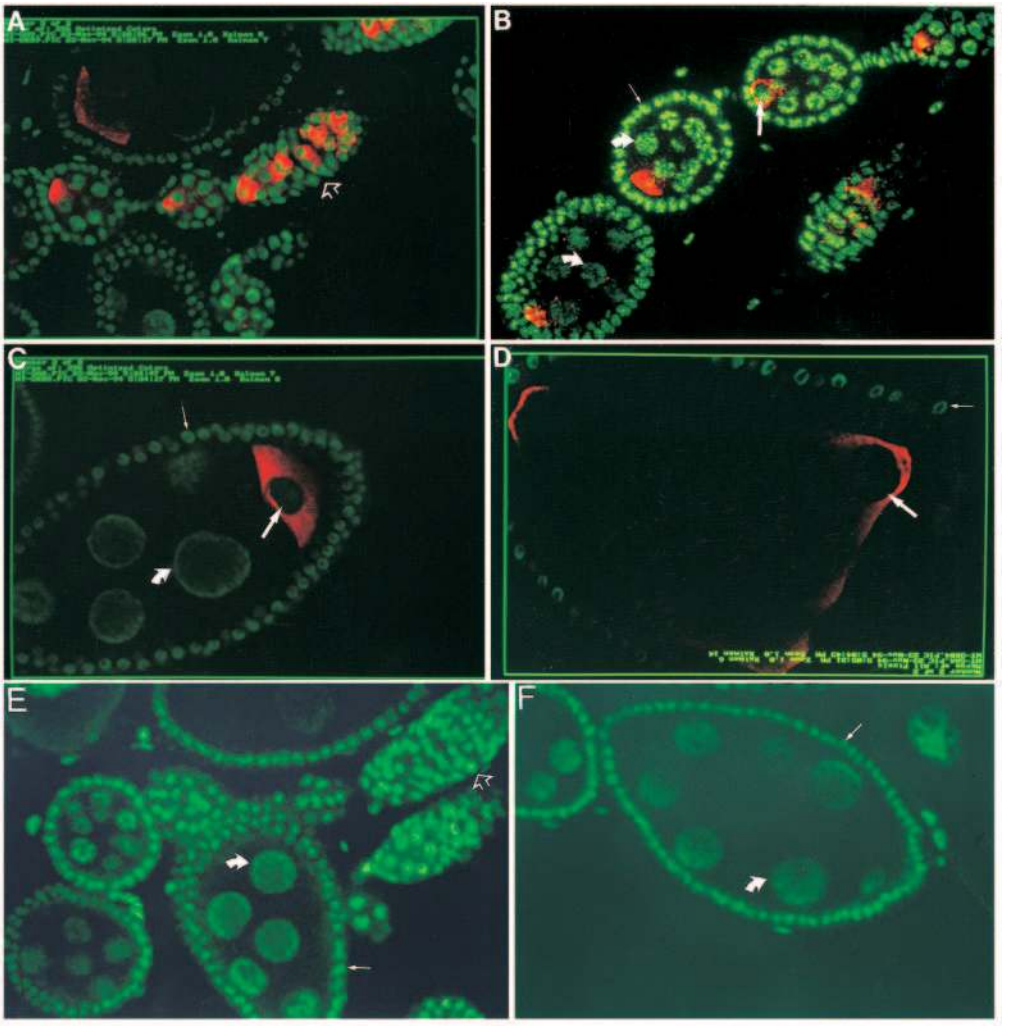

Fig. 2. Expression of GAGA protein in the female germline. Wild-type ovaries were stained with a monoclonal antibody against Orb (red) and a polyclonal antibody against GAGA (green). Orb provides a marker for the oocyte (Lantz et al., 1994) as it localizes to the cytoplasm of the oocyte in region 2 of the germarium (A). In the germarium (open arrow, A) GAGA is present in the nucleus of both germline and somatic cells. Posterior to the germarium are two egg chambers; a stage 1-2, and a stage 4-5. GAGA is seen in nurse cell and follicle cell nuclei. The oocyte nucleus is not in the focal plane. (B) Egg chambers from approximately stage 1 (top right) to approximately stage 6 (bottom left). GAGA is observed in the oocyte nucleus (long arrow), in follicle cell nuclei (thin arrow) and in nurse cell nuclei (curved arrow). Note the progressive reduction in the level of GAGA protein in nurse cell nuclei as the egg chambers develop. (C) Stage 7 egg chamber. Little GAGA is observed in the oocyte nucleus (long arrow). It is reduced in the nurse cell nuclei and appears to be restricted to a peripheral ring (curved arrow). (D) Stage 10B chamber; while GAGA is clearly visible in the somatic nuclei (thin arrow), no GAGA is detected in the oocyte nucleus (long arrow) or ooplasm. Orb protein is found in the oocyte. E and F show GAGA protein in Trl^{13C} ovaries. A Trl^{13C} germarium is indicated by the open arrow in E, while the curved arrow shows the protein pattern in a nurse cell nucleus of a stage 5 or 6 egg chamber.

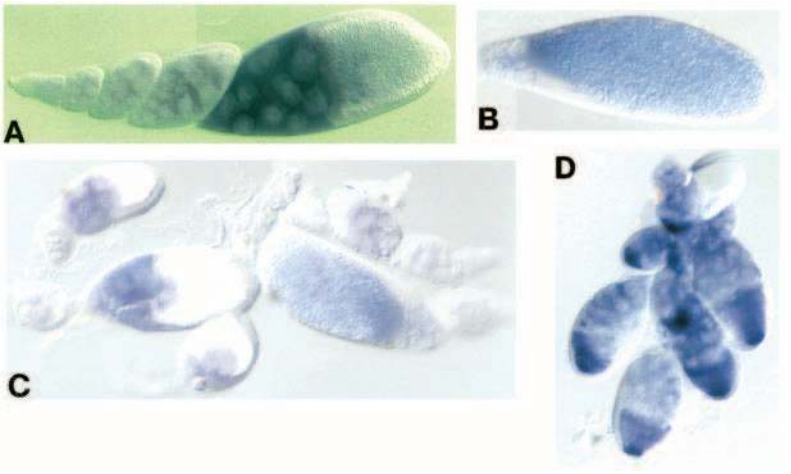

Fig. 3. Expression of *Trl* RNA in wild-type and Trl^{13C}mutant ovaries. A and B show the hybridization pattern in whole mounts of wild-type ovaries probed with a *Trl* cDNA. In C and D whole mounts of Trl^{13C} ovaries were probed, respectively, with a *Trl* and an *oskar* cDNA.

Trl RNA continue to accumulate in the nurse cells until they degenerate around stages 11-12 (Fig. 3B). At this time, the *Trl* RNA appears to be dumped into the oocyte.

The overall pattern of *Trl* RNA accumulation in Trl^{13C} ovaries is quite similar to wild type (see Fig. 3C). Like wild type, there is an apparent increase in *Trl* RNA expression after the onset of vitellogenesis, and this RNA accumulates in the nurse cells until it is dumped into the oocyte around

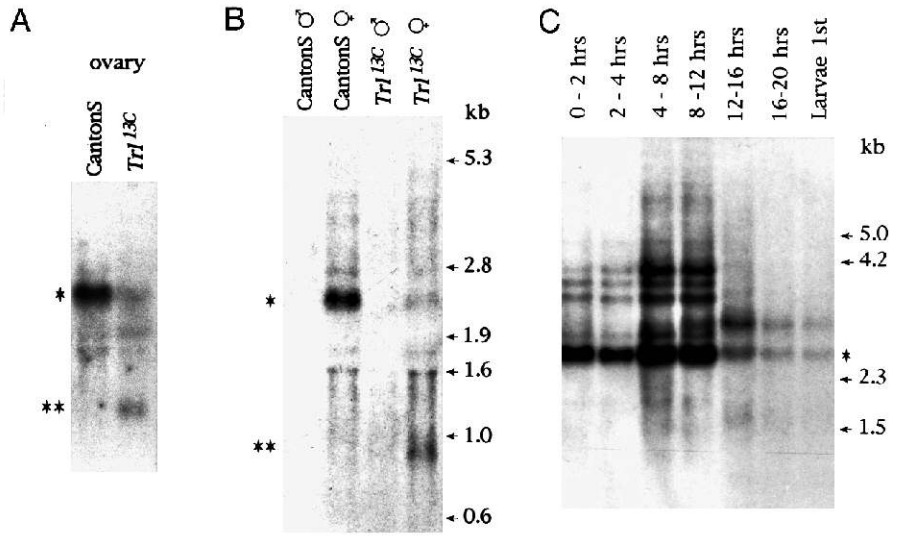

Fig. 4. Northern analysis of *Trl* mRNA. A and B show northern blots of RNA prepared from wild-type and Trl^{13C} flies. (A) An autoradiograph of a northern blot of total RNA from wild-type and Trl^{13C} ovaries. The autoradiograph in B is a northern blot of total RNA prepared from wild-type and Trl^{13C} adult males and females. C is a developmental northern blot of poly(A)$^+$ RNA prepared from wild-type animals. *, major *Trl* mRNA in wild type; **, aberrant *Trl* mRNA in Trl^{13C}.

stages 11-12. However, the amount of *Trl* RNA expressed in Trl^{13C} chambers during vitellogenesis appears to be less than in wild type, and we consistently observe a weaker hybridization signal in the mutant chambers. This reduction does not seem to be due to a general defect in either RNA expression or localization, since the pattern of accumulation of another maternal mRNA, *oskar*, appears normal (see Fig. 3D).

Though these experiments indicated that the amount of *Trl* RNA deposited into Trl^{13C} oocytes is less than in wild type, it was not clear whether this reduction is sufficient to account for the maternal effect lethality. For this reason, we analyzed the *Trl* mRNA profile by northern blot analysis. In wild-type females and ovaries (Fig. 4A,B), we detect one major mRNA of 2.4 kb, and three larger minor species. These four RNAs are deposited and stored in the egg, and are observed both in unfertilized eggs (not shown), and in 0- to 2-hour and 2- to 4-hour embryo collections in roughly the same relative abundance (Fig. 4C and Soeller et al., 1993). The *Trl* mRNA profile in wild type animals changes at 4 hours, and there is an apparent increase in levels of the larger species, while a very weakly labeled 3.0 kb RNA becomes much more prominent. This change in the RNA profile presumably marks the time in development when *de novo Trl* expression first begins (see Soeller et al., 1993). Between 12 and 20 hrs the level of *Trl* mRNA begins to drop and it then remains at a relatively low level until expression is again elevated in the adult female germline.

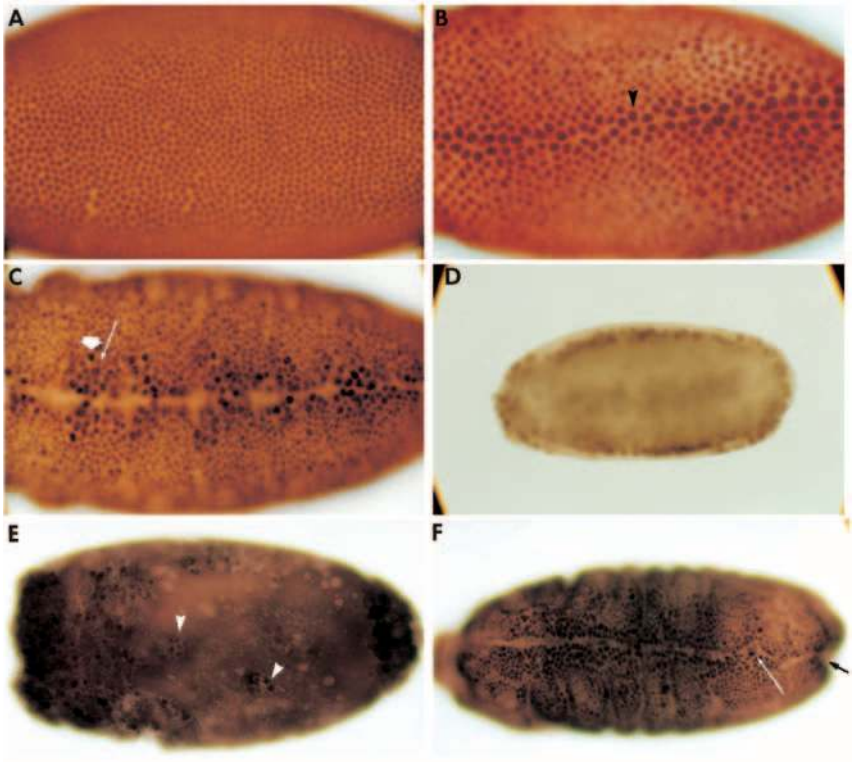

Fig. 5. Trl^{13C} embryos show variable levels of GAGA protein. (A-C) Wild-type embryos; (D-F) Trl^{13C} embryos. (A) All nuclei of blastoderm embryos have GAGA protein. (B) During gastrulation cells along the ventral midline (see arrowhead) typically have higher levels of GAGA protein than their neighbors. (C) Later during neurogenesis, different cells in the CNS appear to have a characteristic level of GAGA protein (compare thick and thin arrows). D: This blastoderm Trl^{13C} embryo has an uneven distribution of GAGA protein (and a reduced number of nuclei on the surface). E: In this gastrulating Trl^{13C} embryo, GAGA is found in small patches of cells (see arrowheads). F: This germband retracting embryo has a nearly wild-type GAGA protein distribution; however, there are regions of the embryo in which GAGA is missing or present in low levels in clusters of cells. The posterior of the embryo shows some developmental abnormalities (arrows).

As can be seen in Fig. 4A,B, the pattern of *Trl* RNA in Trl^{13C} mutant females is quite different from that of wild type females. First, Trl^{13C} females and ovaries have substantially reduced amounts of the major 2.4 kb *Trl* mRNA. Second, we observe abundant quantities of a much shorter RNA species of around 1.0 kb in length. This RNA presumably arises from the aberrant processing of Trl^{13C} transcripts containing the P-transposon insertion and would be too small to encode the major GAGA protein species. Thus, although the level of GAGA RNA expression increases at the onset of vitellogenesis in Trl^{13C} egg chambers as in wild type, our northern blot results indicate that much of this RNA is likely to be non-functional.

Expression of GAGA protein in wild type and *Trl* mutant embryos

The failure to deposit a full complement of wild-type maternal mRNAs would be expected to result in abnormalities in GAGA protein expression in embryos produced by Trl^{13C} mothers. To determine if this is the case we examined the pattern of expression of GAGA protein in wild-type and mutant embryos by whole-mount immunostaining. Low levels of GAGA protein can first be detected in wild-type embryos (or embryos from females heterozygous for the Trl^{13C} lesion) during the very early cleavage cycles, suggesting that the translation of maternal mRNA commences shortly after egg laying. The amount of GAGA protein increases substantially during the early nuclear cleavage cycles, and by the blastoderm stage quite high levels of nuclear protein are observed (Fig. 5A). While all somatic and pole cell nuclei of the blastoderm appear to have equivalent levels of protein, this is not the case at later stages of embryogenesis. As can be seen in Fig. 5B, the cells along the ventral furrow appear to have higher amounts of protein. Later, as the CNS begins to develop, there are differences in the level of GAGA protein in different neuroblasts (NB), ganglion mother cells (GMCs) and glial cells (Fig. 5C).

As anticipated, GAGA protein expression in embryos from Trl^{13C} mothers is abnormal. Several different expression patterns are observed, and these presumably correspond to the different phenotypic classes described above (see Table 1). In class I embryos, which arrest development prior to cellular blastoderm formation, we typically detect little GAGA protein (Fig. 5D). Embryos in the second class cellularize, but subsequently show defects in gastrulation and other major developmental abnormalities. While GAGA is found in these embryos, the amount of protein is generally reduced and there are large patches which have no protein (Fig. 5E). Embryos in the third class develop farther and typically exhibit subtle patterning defects. For example, the embryo shown in Fig. 5F has defects in the posterior and in the midline region. While most nuclei have GAGA, the level of protein in different nuclei is more heterogeneous than in wild type. In addition, there are often small islands of nuclei which have little protein. A similar variability in the level and distribution of GAGA protein was observed in embryos produced by females *trans*heterozygous for Trl^{13C} and the Trl^{62} allele (not shown).

Early embryonic divisions are affected in *Trl* embryos

A plausible hypothesis to explain the early arrest of the class I Trl^{13C} embryos is that some step in the nuclear division cycle is defective in the absence of GAGA. To explore this possibility we examined the nuclear division cycles in embryos from Trl^{13C} mothers. Although some abnormalities were observed in the nuclear cleavage cycles of pre-syncytial blastoderm embryos, these are most clearly visualized after nuclear migration, during cycles 10-12, when a substantial fraction of the Trl^{13C} embryos exhibit readily detectable defects in nuclear division. Several types of abnormalities are observed. The first is an asynchrony in the nuclear division cycle. In wild-type embryos, the nuclear division cycles are synchronized until cellular blastoderm formation. However, the nuclei at the poles are slightly advanced relative to those in the central region, and in the wild-type embryo shown in Fig. 6A, the nuclei at the poles have just entered metaphase while those in the center are still in prophase. In many of the Trl^{13C} embryos the nuclear division cycles become asynchronous during the syncytial blastoderm stage. This is illustrated by the embryo shown in Fig. 6B. In this embryo, nuclei in interphase (arrowheads) are located adjacent to nuclei that are in prophase (thick arrows) or have entered metaphase (thin arrow) or have already divided. Another defect is in chromosome segregation during anaphase. As can be seen in Fig. 6C, many of the nuclei in this Trl^{13C} embryo are joined by elongated chromatin bridges, often with unequal amounts of chromatin pulled to opposite poles. Some of the connected nuclei have a dumb bell shape suggesting that the chromatin may have already begun to decondense even though chromosome segregation is not properly completed. These abnormalities would be consistent with a failure in the separation or decatenation of the paired homologs at the beginning of anaphase. Finally, there is evidence of extensive chromosome fragmentation. As illustrated in Fig. 6D-F, considerable heterogeneity in nuclear size can be observed. Some nuclei are abnormally small (small arrows in Fig. 6E) and there are also many weakly stained chromatin clumps distributed irregularly in the cytoplasm. Other nuclei in these embryos are much larger than normal (large arrow). These nuclei appear to have excess DNA, perhaps because of the unequal segregation of chromosomes to daughter nuclei and/or the inappropriate activation of a replication cycle without properly completing mitosis. This spectrum of abnormal nuclei would suggest that the defects in chromosome segregation evident in Fig. 6C may ultimately result in the disruption of the chromosomes.

Evidence that these abnormalities in nuclear division are likely to be due to insufficient amounts of GAGA protein comes from the staining of mutant embryos with GAGA antibodies. Previous studies on wild-type embryos by Raff et al. (1994) indicate that GAGA protein is not distributed uniformly along the chromosome during the nuclear cleavage cycles. Instead, as illustrated in Fig. 7A-C, much of the protein is concentrated in centromeric regions. The centromeric heterochromatin of *D. melanogaster* contains satellite DNA consisting of reiterated GAGA binding sites (AAGAGAG and AAGAG; Lohe et al., 1993), and these satellites comprise more than 7% of the genome. Raff et al. (1994) have shown that the GAGA protein is bound to these satellite sequences throughout the cell cycle in syncytial blastoderm embryos. This would suggest that GAGA initially binds to the chromosomes while chromatin is being re-assembled after replication and then remains associated as the chromosomes progress through the mitotic cycle of condensation, segregation and decondensation.

Heterochromatin-associated GAGA protein is not observed

in Trl^{13C} embryos exhibiting nuclear division defects. Shown in Fig. 7D is a Trl^{13C} embryo stained with histone antibody. This embryo has a heterogeneous collection of abnormal nuclei and chromosome fragments (see arrows) typical of that found in embryos showing severe nuclear division defects (Fig. 6). As can be seen in Fig. 7E and F, little or no GAGA protein can be detected in the abnormal nuclei of this embryo. Further evidence that the nuclear division defects are likely to be caused by a reduction in GAGA protein comes from Trl^{13C} embryos in class II or class III. An example of such an embryo, double stained with antibodies against GAGA and histone, is shown in Fig. 7G-I. Most nuclei in this embryo are near the end of prophase or have entered metaphase. They have a morphology similar to wild-type embryos (compare Fig. 7A-C) and have readily detectable levels of GAGA. There are, however, two giant nuclei or 'supernova' (arrows in Fig. 7G) in which the chromosomes appear to be in a completely decondensed state. Judging from the very strong staining of these 'supernova' with histone antibody (Fig. 7G) it would appear that the chromosomes have completed (at least one round of) replication, but are unable to initiate the condensation process at the beginning of prophase. Significantly, unlike the normal nuclei, the 'supernovas' have little or no GAGA protein. This can be seen in Fig. 7H which shows the staining pattern obtained with GAGA antibody (see arrows) and in the merged image in Fig.7I. Similar results were obtained for embryos from Trl^{13C}/Trl^{62} mothers.

Trl^{13C} embryos display abnormalities in gene expression

engrailed

GAGA was originally identified in in vitro transcription experiments using promoter fragments from the *en* and also the *Ubx* gene (Soeller et al., 1988; Biggin and Tjian, 1988). The *en* promoter has seven GAGA binding sites just upstream of the transcription start, and in Schneider cell transfection assays these sites are found to be important for maximal expression (Soeller et al., 1988). An obvious question is whether these GAGA sites (or other sites in the 35 kb *en* regulatory region) are also important for *en* expression during embryogenesis. As illustrated by the wild-type germ band extended embryo in Fig. 8A, *en* is expressed in 15 stripes in the ectoderm (arrow) and neuroectoderm (arrowhead). Abnormalities in this pattern of expression are evident in Trl^{13C} embryos. In the class II embryo shown in Fig. 8B-C there is a severe disruption in the segmentation process; the embryo is shorter than wild type and gastrulation is abnormal. This embryo also displays severe defects in *en* expression. Except for weak expression near the anterior of the embryo, the ectodermal *en* stripes are almost completely absent. In addition only part of the normal CNS expression pattern is evident (thin arrow in Fig. 8B and arrowheads in Fig. 8C).

Defects in the *en* expression pattern are also observed in many of the class III embryos. Although the segmentation process in the class III embryo in Fig. 8D is less severely disrupted than in the class II embryo in Fig. 8B-C, there are still abnormalities in *en* expression. While all of the stripes seem to be present in this germ band extended embryo, within each stripe there is a 'mosaic' pattern of *en* expression. Thus, although many of the cells in the stripe appear to express normal levels of En, they are often adjacent to cells expressing little or no protein. Moreover, in some instances large gaps in the stripes can be seen (see arrow).

To ascertain whether these defects in *en* expression are likely to be due to reductions in GAGA, we examined Trl^{13C} embryos double stained with En and GAGA antibodies. As illustrated by the class III embryo in Fig. 8E-G, there is generally a good correlation between the level of *en* expression and the presence of GAGA protein. In nuclei (see short arrows in Fig. 8E,F) which stain strongly with the *en* antibody, we observe comparatively high levels of GAGA protein, while nuclei which stain weakly with *en* antibody (long arrows) have comparatively low levels of GAGA protein. Moreover, in regions of the embryo where the *en* stripe is interrupted, little or no GAGA protein can be detected.

fushi tarazu

Like *en*, the *ftz* promoter region contains potential binding sites for the GAGA factor. One of these is close to the transcription start site, while there are three additional sites located approx. 350 bp upstream. In embryos from wild-type mothers, *ftz* is initially expressed at the syncytial blastoderm stage in a broad band which quickly resolves into 7 transverse stripes in even numbered parasegments. After the onset of gastrulation these stripes begin to fade, and by mid-gastrulation have disappeared. Later during germ band shortening *ftz* is expressed in the developing CNS. Using antibodies direct against Ftz protein we observed two different types of defects in *ftz* expression in those Trl^{13C} embryos which progress to the blastoderm stage. In the first, which is the most extreme case, the seven *ftz* stripes are not readily detectable (not shown). These embryos typically appear to have little or no GAGA protein. In the second, less extreme case, stripes of Ftz protein can be observed; however, within the stripes there is a 'mosaic' pattern of expression with some cells expressing relatively high levels of protein, and other cells expressing little or no protein. This mosaic pattern of expression is illustrated by the embryo shown in Fig. 9. Like *en*, GAGA protein is typically found in those cells in the stripe that have relatively high levels of *ftz* expression (Fig. 9B-C). By contrast, cells that have reduced levels of *ftz* expression, have little or no GAGA.

DISCUSSION

Although molecular and biochemical studies suggest that the *Drosophila* GAGA factor may play a critical role in the transcription of many developmental and house-keeping genes, animals homozygous for presumed null alleles in *Trl* (the GAGA gene) survive to the larval stage and exhibit no readily apparent developmental defects (Farkas, et al. 1994). One explanation for this finding is that sufficient quantities of *Trl* gene products are deposited in the egg during oogenesis to sustain the mutant animals through the larval phase. This explanation is supported by our analysis of the expression of the *Trl* gene during oogenesis in wild-type females and in females homozygous for the semi-vital hypomorphic *Trl* allele, Trl^{13C}.

In wild-type females, high levels of one major 2.4 kb *Trl* mRNA and several larger minor RNAs are deposited in the developing oocyte where these RNAs are stored, apparently in a masked form, until after egg deposition. In Trl^{13C} females

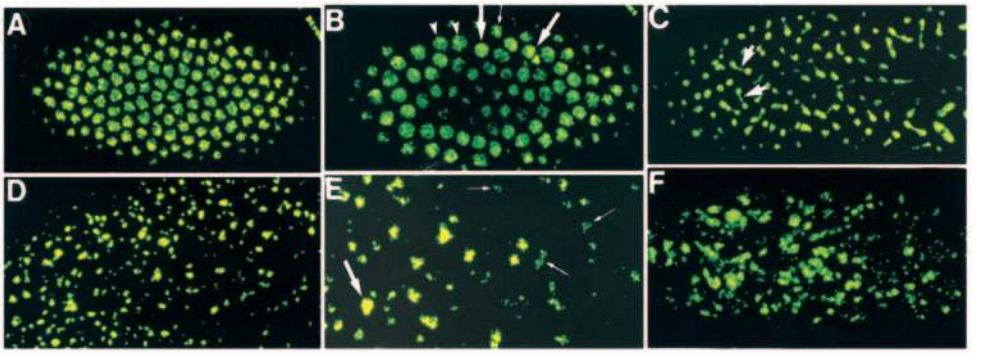

Fig. 6. *Trl*^{13C} embryos show nuclear defects during nuclear cleavage divisions. Embryos in A-F were stained with an antibody against histone 2A and examined by confocal microscopy. (A) A wild-type embryo in approx. nuclear cycle 12 and (B) an embryo from *Trl*^{13C} mothers of approximately the same stage. In B, nuclei which are in different stages of the mitotic cycle can be observed. The arrowheads indicate nuclei in interphase, the large arrows indicate nuclei in prophase, and the thin arrow shows a metaphase nucleus. In C, a slightly older *Trl*^{13C} embryo is shown. In this embryo chromosome segregation is abnormal, and many nuclei are connected by chromosomal bridges. In the embryos shown in D and F, there appears to be extensive chromosomal breakdown, and a very heterogeneous staining pattern is observed. E shows an enlargement of the embryo in D. As indicated by the thin arrows many of the remaining 'nuclei' appear to have only an incomplete complement of chromosomal DNA and exhibit much reduced levels of staining. Others appear much larger than normal (large arrow). Similar nuclear division defects were obtained for embryos produced by mothers *trans*heterozygous for *Trl*^{13C} and *Trl*^{62}.

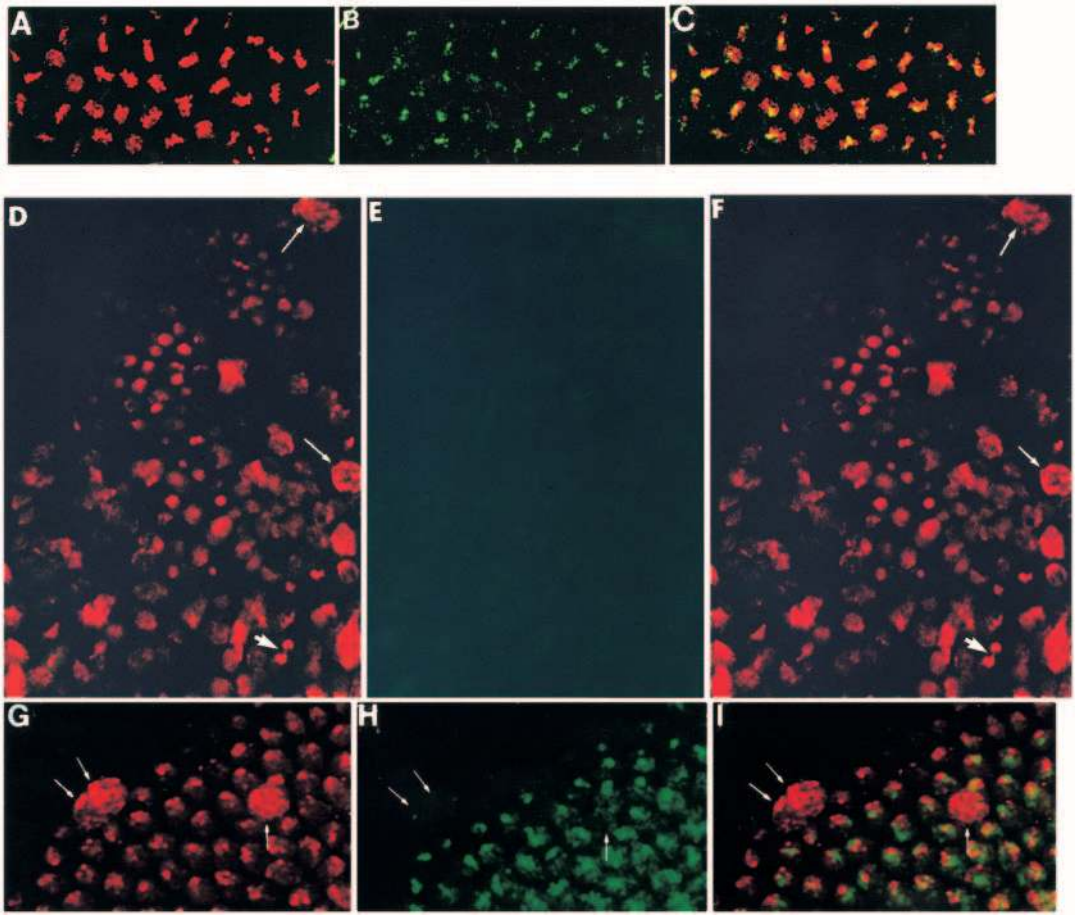

Fig. 7. Nuclear division defects are correlated with reduced levels of GAGA protein. (A-C) Embryos produced by *Trl*^{13C}*/TM2* mothers were double stained with GAGA antibody (B, green) and propidium iodide (A, red) and analyzed by confocal microscopy. (C) A merged image of the propidium iodide and GAGA antibody stained embryo. GAGA protein is localized in several small dots in heterochromatin in these mitotic nuclei (see also Raff et al., 1995), which appear yellow in the merged images. (D-I) Embryos produced by *Trl*^{13C}mothers were double stained with antibodies against core histone (D,G) and GAGA (E,H) proteins. (D-F) A mutant embryo exhibiting severe nuclear division defects like the embryos shown in Fig. 6D-F. (G-I) A class II or class III mutant embryo in which most nuclei appear to be relatively normal. However, two supernovas can be seen in this region of the embryo (small arrows). These supernovas have little or no GAGA protein (see H and the merged image in I).

the expression of these maternal mRNAs is defective. There is a marked reduction in the level of the major 2.4 kb RNA, and instead an aberrant 1.0 kb *Trl* mRNA are found. Since the *Trl*^{13C} mutation is due to the insertion of a large P-element transgene into the first intron of the *Trl* gene, it seems likely that this foreign DNA disrupts the proper processing of the *Trl* transcripts.

Although the major maternal contribution of the *Trl* gene appears to be masked mRNA, relatively high levels of GAGA protein are present in the germ cells of the ovary, particularly

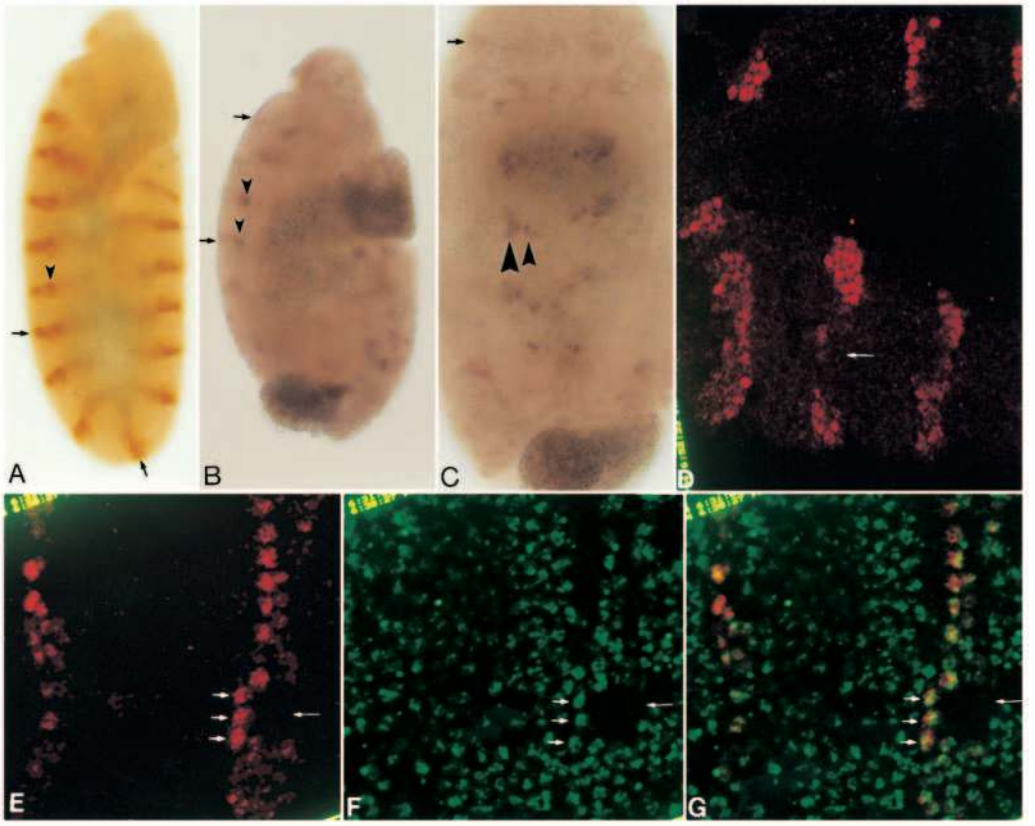

Fig. 8. Trl^{13C} mutation affects the expression of engrailed gene. (A) A wild-type embryo stained with *en* antibody. The ectodermal stripes are marked with arrows and the CNS expression is shown with an arrowhead. (B,C) A gastrulating Trl^{13C} embryo from different angles. Embryogenesis is severely disrupted, and the *en* stripes are almost completely missing. Two weakly labeled stripes can be seen in the ventrolateral ectoderm near the anterior (C, arrow). Some residual CNS expression is also evident. (In B it is indicated by the two arrowheads while in C the large arrowhead marks L neurons and the smaller arrowheads ML neurons). (D) A germ band extended Trl^{13C} embryo with only moderate developmental defects. Most of the *en* stripes are present in this embryo; however, many stripes have cells which express little or no En protein (see arrow). E-G is a Trl^{13C} embryo double stained with En (E, red) and GAGA (F, green) antibodies. As can be seen in E, the En stripes in this embryo are incomplete or have gaps. Some cells have En protein (short arrows) while others have little or none (long arrow). The cells expressing En have GAGA protein in their nuclei (short arrows), while the cells which have little or no En, lack GAGA. (G) A merged image from E and F. Although *en* expression in these mutant embryos is tightly correlated with the presence and level of GAGA protein, we infrequently observed a single nucleus which stained with the En antibody, but appeared to have only rather low levels of GAGA protein (not shown). The reason for these exceptional nuclei is not clear. It could reflect stochastic differences in the chromosomal distribution of limiting amounts of GAGA protein. Alternatively, there may only a transient requirement for GAGA function, after which the protein is dispensable.

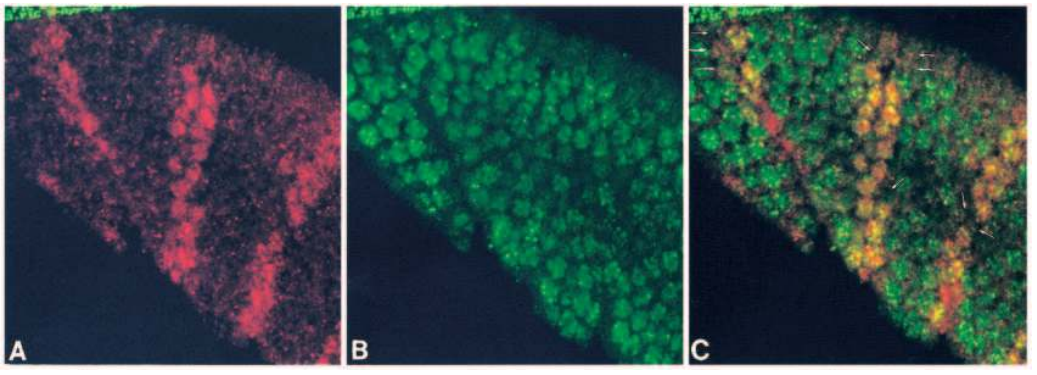

Fig. 9. *ftz* expression is also affected in Trl^{13C} embryos. Shown here is a portion of a germband extended Trl^{13C} embryo, double stained with *ftz* (A, red) and GAGA (B, green) antibodies and examined by confocal microscopy. In C, the two images are merged. Those cells that are not expressing *ftz* are also lacking GAGA protein (arrows), giving a mosaic pattern. More extreme effects of *Trl* mutation on *ftz* expression was also observed (see text).

in the cells that populate the germarium: the stem cells, the dividing cystocytes and the newly formed 16-cell cysts. During these early stages, all of the germ cell nuclei stain intensely with GAGA; however, shortly after the stage 1 egg chamber buds off from the germarium, this pattern of accumulation undergoes two major and unexpected alterations. The first is the virtual disappearance of GAGA protein from the oocyte nucleus, and this occurs well before the onset of vitellogenesis. The second is the gradual reduction in GAGA protein in the nurse cell nuclei. As the egg chamber development progresses from stage 1 to the middle of vitellogenesis, the nurse cell chromosomes undergo multiple rounds of endomitotic replication, increasing to as many as 1000 copies. Over this same period the intensity of GAGA antibody staining does not increase but instead becomes weaker, ultimately dropping to background levels. It seems likely that there is little or no *de novo* GAGA protein accumulation following the formation of the stage 1 chamber. Thus the gradual reduction in antibody

staining could be due both to a dilution of the protein as the nurse cell nuclei grow in size and to active degradation. Additionally, there is a redistribution of the GAGA protein to sites around the periphery of the nuclei. This unusual localization is particularly evident in early vitellogenic egg chambers (stages 7-8) and suggests that GAGA is no long associated with bulk chromatin at this point in oogenesis.

In this context, it is interesting to note that the reduction and relocalization of GAGA protein between stages 1 and 8 is accompanied by an alteration in the inducibility of the *hsp70* genes (which have GAGA bindings sites in their promoters) in nurse cell nuclei (Ambrosio and Schedl, 1984). In early egg chambers, where the level of GAGA protein is still high, the nurse cell *hsp70* genes are fully inducible. However, as the GAGA protein level drops, there is a concomitant loss in inducibility and by stage 7 the nurse cell *hsp70* genes are refractory to induction. By contrast, the *hsp83* gene, which does not contain GAGA sites in its promoter is still expressed at a high level in older chambers.

While there is a good correlation between the inducibility of the *hsp70* genes and the presence of GAGA, this correlation does not hold for another set of repeated genes, the histone H3 and H4 genes, which also have GAGA binding sites in their promoters (Gilmour et al., 1989). There are two phases of histone mRNA expression during oogenesis (Ambrosio and Schedl, 1985). In phase I, which extends from stage 1-10, histone RNA synthesis is coupled to the endomitotic replication cycles of the nurse cell nuclei. During this phase, histone mRNA accumulates transiently in only a few cells in a chamber at any one time (because replication is asynchronous), and the RNA is not deposited in the oocyte. In phase II, which begins at stage 10B, histone mRNA synthesis is uncoupled from the replication cycle and the histone genes in all 15 nurse cells are expressed at high levels. The mRNA synthesized during this phase is dumped into the oocyte for storage as maternal message. Although high levels of GAGA are present at the beginning of phase I (stage 1), the level drops throughout this phase and GAGA can no longer be detected in nurse cell nuclei when phase II of histone mRNA synthesis is initiated. Taken together these findings would suggest that GAGA is unlikely to be required for the expression of the histone H3 and H4 genes during oogenesis. It is possible that some other factor(s) substitutes for GAGA in this very specialized cell type. Alternatively, the GAGA factor may not be essential for the formation or maintenance of a hypersensitive site in the H3-H4 promoter or for the expression of these two genes. In support of this possibility, O'Brien et al. (1995) have recently reported that unlike the *hsp70* heat shock genes, there is little GAGA protein bound to the histone repeat unit in vivo in tissue culture cells (which are of somatic origin).

GAGA factor is required for the pre-cellular blastoderm nuclear division cycles

Perhaps the most unexpected and striking of the MEL phenotypes observed in embryos from Trl^{13C} mothers are the abnormalities in nuclear division. The spectrum of defects range from asynchrony in the nuclear division cycle to chromosome fragmentation and nuclear disintegration. A critical issue is whether these abnormalities in nuclear division reflect a direct requirement for GAGA in some aspect of chromosome structure and/or function, or whether they arise by an indirect mechanism.

The most plausible indirect mechanism is that GAGA is required for the expression of some gene(s), either in the zygote or during oogenesis, whose product is essential for nuclear division. However, we believe that an indirect mechanism of this sort is unlikely. Since zygotic transcription is not required for the proper execution of the nuclear division cycles (Edgar et al., 1986) the mitotic defects cannot be a secondary consequence of failing to transcribe some key gene in mutant embryos. Although we cannot conclusively rule out the possibility that gene products critical for nuclear division in the embryo are miss expressed in Trl^{13C} ovaries, the available evidence would argue against this explanation. First, the temporal pattern of GAGA protein accumulation during oogenesis is inconsistent with a role in the synthesis of abundant macromolecules (such as maternal mRNAs encoding major chromosomal constituents) that are required for the nuclear division cycles in the embryo. The nurse cells become most actively engaged in the synthesis of macromolecules for deposition in the oocyte around stage 7 or 8 just at the time when the level of GAGA protein declines to virtually nothing. Second, the pattern of accumulation of GAGA protein in Trl^{13C} egg chambers is rather similar to that observed in wild type. Thus, even if the GAGA protein were required for the synthesis of a 'critical' gene product at an earlier stage of oogenesis, such a product should be expressed in the Trl^{13C} egg chambers.

While this indirect mechanism seems unlikely, there is evidence suggesting that GAGA protein may actually have a direct role in chromosome structure/function in the early embryo. The first is its localization. GAGA is found associated with the chromosomes of early embryonic nuclei, and this association is maintained throughout the mitotic cycle. The second is the close correlation between nuclear division defects and reductions in level of GAGA protein. Our double labeling experiments indicate that the nuclei in Trl^{13C} embryos which exhibit nuclear division defects typically have little or no detectable GAGA protein.

If GAGA factor plays a direct role in nuclear division, when is it required in the mitotic cycle? Given its postulated role in forming or maintaining nucleosome free regions of chromatin, one might imagine that the GAGA factor would be required for the decondensation of the chromatin fiber once mitosis is complete, remodeling the chromosome so that it is competent for replication or transcription. Although a function in decondensation at the beginning of interphase seems plausible, the abnormalities in nuclear division suggest that the GAGA factor is (also) required at earlier points in the mitotic cycle. The most likely steps are in the condensation and decatenation of the chromosomes following the completion of replication and entry into prophase. To explain the observed nuclear division abnormalities, we suppose that chromatin assembled on newly replicated DNA in the presence of limiting amounts of GAGA would not be a suitable substrate for the processes involved in either the condensation of the chromosome or in the decatenation of the intertwined homologs produced by replication. Under conditions in which there are sub-optimal amounts of GAGA the only consequence might be to prolong these two processes, lengthening prophase and accounting for the asynchronous nuclear divisions noted in many *Trl* embryos. Further reductions in GAGA factor would have more severe conse-

quences. In some cases the nuclei might simply arrest after chromosome replication, perhaps giving rise to the supernovas. In other cases, the nuclei would enter metaphase even though they have not completed chromosome condensation or homolog decatenation. These chromosomes would be unable to segregate, leading first to the formation of chromatin bridges containing lagging chromosomes and ultimately to chromosome fragmentation as the mitotic process is driven to completion.

Since most of the GAGA in precellular blastoderm nuclei is associated with centromeric heterochromatin, it seems likely that the observed defects in nuclear division are related to this particular localization in the chromosome. The targets for GAGA in the heterochromatin are two simple sequence satellite DNAs that make up a substantial fraction of the *D. melanogaster* genome (Raff et al., 1994). Although nothing is known about the chromatin structure of these particular satellites, other centromeric satellite sequences in *Drosophila* are organized into 'phased' nucleosome arrays (Kas and Laemmli, 1992). Since GAGA is capable of positioning nucleosomes in vitro, it would be reasonable to suppose that it may be required in cleavage stage nuclei to properly organize the chromatin architecture of these two simple sequence satellite DNAs. In Trl^{13C} embryos, where GAGA is limiting, these satellite DNAs might be assembled into inappropriately packaged nucleosome arrays that provide little or no accessibility to the factors which mediate condensation and decatenation (e.g. topoisomerase II). Since these satellite sequences are arranged in very large blocks, this could result in extensive chromosomal segments that are incompletely condensed and/or decatentated. Consistent with the idea that defects in heterochromatin structure may cause disruptions in the mitotic cycle, Kellum and Alberts (1995) have found that mutations in the heterochromatin protein HP1 cause a spectrum of nuclear cleavage cycle defects similar to those observed in Trl^{13C} embryos.

On a more general level, the nuclear cleavage cycle defects observed here raise questions about the biological role of heterochromatic satellite sequences. While virtually all higher eukaryotes have heterochromatic regions composed of extensive arrays of simple repeating sequences, the extremely poor conservation of these sequences during evolution has led to the idea that they may be selfish DNAs of no functional significance to the organism (see Doolittle and Sapienza, 1980; Orgel and Crick, 1980). However, our findings (and also the studies of Kellum and Alberts on HP1) raise the possibility that the simple sequence arrays in heterochromatin play an important role in the mitotic process. This view is supported by studies of Murphy and Karpen (1995) on the transmission of the *Drosophila* mini-chromosome deleted for satellite DNA. One plausible role for the satellite DNA would be to help stabilize homolog pairing until decatenation (of the satellite chromatin) is initiated at the onset of anaphase. The only requirement for such a function would be that these heterochromatic sequences are simple repeats, and that the cell contains proteins like GAGA which recognize the repeat sequence and establish appropriate chromatin structures. In this context, it is interesting to note that the GAGA protein is not associated with the centromeric heterochromatin of *D. virilis* (Raff et al., 1995), a species whose satellites do not consist of reiterated GAGA binding sites (Gall and Atherton, 1974). We would anticipate that, unlike *D. melanogaster*, the nuclear cleavage cycles in *D. virilis* embryos would be comparatively normal in the absence of GAGA. However, we would suppose that there must be some other GAGA-like factor(s) in this species which recognizes the *virilis* satellite sequences and organizes their chromatin.

The GAGA factor is required for transcriptional activity

In addition to the nuclear cleavage cycle defects, Trl^{13C} embryos show abnormalities in the transcriptional activity of two embryonic patterning genes, *en* and *ftz*, that contain GAGA binding sites in their promoter regions. The expression defects differ from those typically observed for mutations in maternal loci that are responsible for controlling the spatial or tissue specific patterns of gene activity in the embryo. Mutations in maternal morphogens like *bicoid* or *dorsal* disrupt the choice of cell fate, shifting the patterns of gene activity from one program to another (for review see St. Johnston and Nüsslein-Volhard, 1992). For example, in the absence of *bcd*, the normal program of gene expression in the anterior of the embryo is disrupted; stripes *en* expression disappear, while *ftz* stripes expand. The Trl^{13C} lesion does not appear to cause similar switches from one program of expression to another. Instead, the effects of Trl^{13C} appear to be more global – disrupting gene expression in a manner consistent with that expected for mutations in a protein which functions as an essential cofactor. This is most clearly illustrated by the range of defects in *en* and *ftz* expression in Trl^{13C} embryos. In the more extreme cases, the *en* and *ftz* stripes are absent or almost completely absent. In less extreme cases, only a few stripes may be missing; however, in contrast to the alterations observed in patterning gene mutations, there does not seem to be any regional specificity in this defect. Even more telling are those cases in which there are gaps or discontinuities within individual stripes. Here, it would appear that the regional signals required to activate the expression of the specific stripe may be present, but that the gene is nevertheless unable to properly respond in some of the cells that constitute the stripe.

As was the case for the defects in the nuclear division cycle, an important question is whether the abnormalities in gene expression in Trl^{13C} embryos are direct or indirect. In favor of a direct effect are the cell by cell gaps or discontinuities in expression patterns. For the mosaic patterns of *en* and *ftz* expression we have shown that there is a close correlation between GAGA protein and gene activity. Both genes are generally expressed in cells that have GAGA protein, while cells that do not express the two genes usually have little or no protein. There is, of course, the possible caveat that *en* or *ftz* may not be expressed in cells that have little or no GAGA protein because these cells are derived from a lineage in which there were 'earlier' GAGA-dependent defects in the expression of a key regulator such as one of the gap genes. Although indirect effects of this sort must occur, we believe that many of the deficiencies in gene expression are directly attributable to insufficient levels of GAGA protein. In this view, the defects in gene expression in Trl^{13C} embryos would occur because promoters or enhancer regions containing essential GAGA binding sites are assembled into nucleosomes and are consequently inaccessible to RNA polymerase or potential positive regulators. It is interesting to note that while some of these

genes have nuclease hypersensitive regions associated with their promoters in early embryos, these hypersensitive regions are not constitutive and disappear at later stages of development (Schedl, unpublished data). This would suggest that the GAGA factor may not be able to act autonomously on all promoters or regulatory elements, but may, in some instances, require additional proteins to specify which DNA segments may ultimately form nucleosome free regions.

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