The *gnu* mutation of *Drosophila* causes inappropriate DNA synthesis in unfertilized and fertilized eggs

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Drosophila melanogaster embryos whose mothers are homozygous for the maternal effect lethal mutation *gnu* (GNU embryos) undergo DNA synthesis but no nuclear division; this leads to the formation of a small number of giant nuclei in the syncytial blastoderm. We have shown previously that many components of the mitotic apparatus are present and functional in GNU embryos, and the primary lesion of the *gnu* mutation has therefore remained obscure. Here, we report that fertilization is not necessary for GNU eggs to develop. Giant nuclei originate from the products of female meiosis, and we see autonomously replicating centrosomes that must be maternally derived. If GNU eggs are inseminated, however, the male pronucleus also undergoes DNA replication. Our observations suggest that the GNU cytoplasm permits DNA synthesis in a relatively unregulated manner. In embryos from females homozygous for *gnu* and the female sterile haploid mutation *mh*, we find replication of DNA derived from the male pronucleus. This contrasts with embryos from mothers homozygous for *mh* alone, in which this does not occur. We propose that the *gnu* gene product participates in the repression of DNA synthesis found in unfertilized eggs.

[Key Words: *Drosophila*; DNA replication; egg activation]

Little is known about the processes that regulate the activation of DNA synthesis and the subsequent onset of the mitotic cycle in fertilized eggs. Although extensive biochemical study, mainly of amphibian eggs, has demonstrated the involvement of several factors, such as maturation-promoting factor (MPF) (Wu and Gerhart 1980; Miake-Lye et al. 1983), the molecular basis for this ubiquitous developmental switch is still unclear. The earliest stages of embryogenesis are easily observed in *Drosophila* eggs, and, coupled with the sophisticated genetic and molecular techniques available, this makes *Drosophila* an attractive organism with which to study these events. In this report we demonstrate that *gnu* gene product of *Drosophila* is involved in the repression of DNA replication that occurs in unfertilized eggs.

gnu is a maternal effect lethal mutation of *Drosophila melanogaster*, which maps on the third chromosome at 42.5 map units. It has the striking phenotype that embryos whose mothers are homozygous for the mutation (GNU embryos) undergo DNA synthesis but no nuclear division. This leads to the formation of a syncytial blastoderm that contains a small number of giant nuclei instead of the normal rapidly dividing diploid nuclei seen in wild-type embryos; GNU embryos never cellularize (Freeman et al. 1986). This phenotype led us to believe that the product of the *gnu* gene participates in the mi-

totic division cycle during embryogenesis. However, we have shown previously that many of the components of the mitotic apparatus in GNU embryos are present and functionally adequate (Freeman et al. 1986), and the primary lesion of the *gnu* mutation therefore remained obscure.

Here, we report the unexpected observation that GNU eggs develop even if unfertilized, to give giant nuclei that originate solely from the products of female meiosis. We have also examined the replication of the parental genomes in GNU eggs, and on the basis of these results, we propose that the *gnu* gene product plays a part in the mechanism that normally represses DNA synthesis in the unfertilized egg. In addition, we have exploited the development of uninseminated GNU eggs to show that there is no requirement for a paternal contribution for the formation of centrosomes in the *Drosophila* embryo.

Results

Abnormal DNA synthesis in GNU eggs

Unfertilized wild-type *Drosophila* eggs are arrested at the end of meiosis II (Doane 1960), so that the four haploid products of female meiosis—the three polar bodies and the pronucleus—are present and can be observed in fixed eggs stained with a DNA-binding fluorochrome, as seen in Figure 1a. An unreplicated nucleus, one of the

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products of female meiosis, is indicated with an arrow, and the inset panel shows a similar nucleus at a higher magnification. The state of the chromatin in these eggs

Figure 1. The nuclei of unfertilized *Drosophila* eggs from wild-type (*a*) and homozygous (*b*) *gnu* mothers. Eggs laid overnight at 24°C by virgin females were fixed and stained with the DNA intercalating dye, Hoechst 33258. (*a*) Wild-type egg in which there is no sign of development. The arrow points to condensed chromatin from the polar bodies, and the inset panel shows a similar structure at a higher magnification (bar represents 10 μ m). (*b*) An unfertilized GNU egg. In contrast to the wild-type egg, considerable DNA replication has occurred, leading to the formation of a giant nucleus. We can see no cytological difference between fertilized and unfertilized GNU eggs. Bar represents 50 μ m.

varies: In some cases (as in Fig. 1*a*), the chromosomes are highly condensed, and in other eggs, they are condensed and diffuse. Two or more of the meiotic products often fuse, so that what is usually observed in practice is between one and four separate nuclei. Eggs laid by virgin females homozygous for *gnu* are strikingly different (Fig. 1*b*). Instead of being developmentally arrested, the female meiotic products undergo DNA synthesis, and the eggs develop giant nuclei; these eggs are cytologically indistinguishable from GNU eggs laid by females that have been allowed to mate with males. This result suggests that the *gnu* mutations lifts the total block to DNA replication that exists in the wild-type unfertilized egg.

The *gnu* mutation also affects the development of the polar bodies, the three haploid products of female meiosis other than the female pronucleus. In the wild-type fertilized egg, the three polar bodies are not destined to participate in further development and show no sign of replication, even though they persist throughout cleavage (Rabinowitz 1941). In contrast, when we examine GNU embryos soon after oviposition, we frequently see that the polar bodies have undergone replication (data not shown), giving rise to giant nuclei in older embryos.

Centrosomes are present in unfertilized GNU eggs

Centrosomes are the organelles that organize the poles of the mitotic spindles. We have observed previously that centrosomes replicate independently of nuclei in fertilized GNU embryos and that they are capable of nucleating microtubules into asters, although spindles per se are not formed (Freeman et al. 1986). The parent that donates the centrosomes needed in embryogenesis varies between species (Schatten et al. 1986). Thus, the mouse embryo acquires centrosomes maternally, whereas the centrosomes in sea urchins are contributed by the incoming sperm. In *Drosophila*, the centrosomal source is unclear, although the centriole, the organelle normally found at the center of the centrosome, appears to be derived paternally (Sonnenblick 1950). We have exploited the development of uninseminated GNU eggs to discover whether a paternal contribution is necessary for centrosome formation in *Drosophila*. The monoclonal antibody Bx63 (Frasch et al. 1986), which recognizes a *Drosophila* centrosome-associated antigen, has been used to label uninseminated GNU eggs by indirect immunofluorescence. Figure 2 shows that centrosomes are found in these eggs, and that these are able to nucleate microtubules into asters that are similar to those seen previously in fertilized GNU eggs (Freeman et al. 1986). This indicates that there is not an absolute requirement for a male contribution to the formation of the centrosomes used during cleavage in *Drosophila*.

Sperm pronuclei can undergo DNA synthesis in GNU eggs

A question that arises from the observation that GNU eggs can develop even in unfertilized is whether the eggs

Figure 2. Unfertilized GNU eggs contain centrosomes and asters. Eggs from virgin *gnu/gnu* females were collected overnight at 24°C and stained by indirect immunofluorescence, using the mouse monoclonal antibody Bx63 against *Drosophila* centrosomes and the rat monoclonal antibody YL1/2 against tubulin. The centrosome (left) and tubulin (right) staining in one of the eggs is shown. Three centrosomes can be seen in the field, and each lies at the center of a microtubule aster. This result indicates clearly that the unfertilized *Drosophila* egg has the capacity to form functional centrosomes without any contribution from the sperm.

are capable of being inseminated and, if so, whether DNA of the sperm pronucleus is then replicated. To address this question, homozygous *gnu* females were mated with males of a transformed strain whose DNA was marked with a bacterial chloramphenicol acetyltransferase (CAT) gene (Gorman et al. 1982). DNA was prepared from embryos collected from this cross and hybridized with radiolabeled probes for the larval serum protein (LSP) 1 α gene, present in DNA from both males and females, or the CAT gene, present only in DNA from the males. This has allowed us to determine unambiguously whether DNA of the male pronucleus replicates in GNU embryos. Figure 3a confirms that the CAT probe does not detect DNA sequences in wild-type or GNU embryos fertilized by males not carrying the CAT gene. Figure 3b shows that when wild-type females are mated with males bearing the CAT gene, the paternal genome replicates as expected (Fig. 3b). In this case, half the DNA in the developing embryo is derived from the male pronucleus, and similar hybridization signals can be seen with the LSP and CAT probes. An equivalent

signal is also seen with LSP and CAT probes when homozygous *gnu* females are mated with CAT-bearing males (Fig. 3b), indicating that the male pronucleus does replicate in GNU eggs. This indicates that although GNU eggs can develop when unfertilized, they can also be inseminated, and when they are, the sperm pronucleus undergoes DNA synthesis, participating in the formation of giant nuclei.

Figure 3. The male pronucleus replicates in inseminated GNU eggs. (a) DNA was isolated from embryos from a cross between wild-type (WT) or *gnu/gnu* (*gnu*) females and males not carrying the CAT gene. The DNA from 250 embryos was divided into two equal parts and probed with a radiolabeled DNA probe containing a 1.6-kb fragment of the *Drosophila* LSP 1 α gene (left panel) or a similarly sized fragment of the CAT gene (right panel). There is no hybridization of DNA of either type of embryo with the CAT probe, demonstrating the absence of CAT sequences in the genome of wild-type or GNU embryos. The LSP hybridization signal is higher in wild-type than in GNU embryos, indicating that although giant nuclei are formed, the GNU embryos do not replicate their DNA to the same extent as fully developed wild-type embryos do. The plasmid control is 5 ng of the parental plasmid from which each probe was isolated. The approximately equal degrees of hybridization of the LSP and CAT probes shows that both were of equivalent specific activity. (b) DNA was isolated from embryos from a cross between males bearing the CAT gene and wild-type (WT) or *gnu/gnu* (*gnu*) females that have no CAT sequences in their genomes. The DNA from 200 embryos (upper band in each pair) and 300 embryos (lower band in each pair) was divided into two equal parts and probed with the LSP (left panel) and CAT (right panel) probes, as above. The CAT probe hybridizes with DNA from both types of embryo, indicating that DNA derived from the male is replicated in both wild-type embryos and GNU embryos.

gnu causes the replication of male-derived DNA in embryos whose mothers are homozygous for a maternal haploid mutation

It seems that GNU cytoplasm permits indiscriminate DNA synthesis in all nuclei within the egg. We tested this idea further by combining the *gnu* mutation in the maternal genome with the X-linked female sterile haploid mutation *mh* (Gans et al. 1975; Zalokar et al. 1975; Santamaria and Gans 1980). Cytological and genetic analysis of embryos from females homozygous for *mh* (referred to here as MH embryos) has shown that the male pronucleus enters the egg but does not participate in syngamy, and that the female pronucleus alone is the source of the haploid cleavage nuclei in MH embryos (Santamaria and Gans 1980; Santamaria 1983). We constructed females homozygous for both *gnu* and *mh* in order to test whether the male pronucleus, which is unable to develop in the cytoplasm of an MH embryo, will replicate its DNA when the *gnu* gene is also mutated. As in the previous experiment, these females were mated with males bearing the CAT gene, and DNA from the resulting embryos was hybridized with an LSP probe and a CAT probe. Figure 4 demonstrates that, as expected, the male genome is significantly underreplicated in embryos resulting from a cross between females homozygous for *mh* alone and CAT-bearing males. However, when CAT-bearing males are mated to females homozygous for *mh* and *gnu*, the resulting embryos, which resemble GNU embryos in phenotype (data not shown), replicate male-derived DNA to approximately the same extent as female-derived DNA (Fig. 4b). Figure 4 includes diagrams of the eggs derived from mothers of both genotypes, intended to clarify the main results of this experiment. The weak hybridization between the CAT probe and DNA from MH embryos from a cross

with CAT-bearing males (Fig. 4a) is probably due to a small amount of aberrant replication of the male pronucleus in the developing MH egg. Previous experiments that have analyzed the *mh* mutation have shown clearly that the male pronucleus does not participate in cleavage (Zalokar et al. 1975; Santamaria and Gans 1980; Santamaria 1983), but do not rule out some such replication. Nevertheless, the ratio of CAT : LSP hybridization is much greater in MH;GNU embryos than in those derived from females homozygous for *mh* alone, indicating that the *gnu* mutation is overriding some block to male pronuclear replication. This provides a further example in which DNA from an embryo nucleus that would not normally replicate does so when the *gnu* gene is mutated.

Discussion

Our results suggest that the wild-type product of the *gnu* gene plays a part in the repression of DNA synthesis that is found in the unfertilized egg. Because a similar block occurs in nearly all sexually reproducing species, its mechanism is of general interest. We have found several separate examples of abnormal DNA synthesis occurring in GNU eggs. First, the DNA of the haploid nuclei derived from female meiosis undergoes replication in GNU eggs that have not been fertilized. Whether or not GNU eggs are fertilized, the three polar bodies, as well as the female pronucleus, undergo DNA synthesis, whereas the polar bodies do not replicate at all in wild-type eggs. Finally, the male pronuclear DNA undergoes replication in fertilized GNU eggs, thereby participating in the formation of giant nuclei. This occurs even when eggs are derived from mothers homozygous for both *gnu* and the female sterile haploid mutation *mh*. In contrast,

Figure 4. The male pronucleus does not replicate in MH embryos but does in MH;GNU embryos. (a) DNA was isolated from three batches of embryos (0–24 hr old) from a cross between *mh/mh* females and males bearing the CAT gene (400, 400, and 300 embryos, from top to bottom). The DNA samples were divided into two equal parts and probed with the radiolabeled probes LSP (left panel) and CAT (right panel), described in the legend to Fig. 3. Only a small amount of hybridization is seen with the CAT gene, compared with the LSP gene, indicating that the male-derived DNA is considerably less replicated than female-derived DNA. A representation of an MH embryo during cleavage is shown; (○) haploid nuclei derived from the female pronucleus; (●) the male pronucleus. (b) DNA was isolated, as described above, from a cross between *mh/mh;gnu/gnu* females and males bearing the CAT gene (230, 160, and 160 embryos, from top to bottom). The DNA samples were divided into two equal parts and probed with LSP (left panel) and CAT (right panel). Equivalent amounts of hybridization are seen with both probes, indicating that male-derived DNA is significantly replicated. A representation of these MH;GNU embryos is shown; (○) Female-derived giant nucleus; (●) male-derived giant nucleus.

fertilized eggs derived from mothers homozygous for *mh* alone, in which the male pronucleus does not participate in syngamy (Zalokar et al. 1975; Santamaria and Gans 1980; Santamaria 1983), show no significant replication of male pronuclear DNA. These cases of inappropriate DNA synthesis suggest that the *gnu* mutation sets up a "replicative environment" in the GNU egg, in which relatively indiscriminate DNA synthesis can occur.

These results are consistent with a model in which DNA replication in the early embryo is normally only possible for a nucleus that has achieved syngamy. This would ensure that the nuclei in the unfertilized egg do not replicate prior to sperm entry and that the polar bodies do not undergo further development, even after fertilization. If *gnu* mutants are defective in a component of the system that is responsible for this repression, we would expect to find the pattern of DNA synthesis that is seen in GNU embryos. This uncontrolled DNA synthesis appears to prevent normal mitosis from being initiated, resulting in the formation of giant nuclei. The details of this model are obviously speculative, but there is good evidence that the *gnu* gene product plays a part in the repression of DNA synthesis in the *Drosophila* egg.

Uninseminated GNU eggs contain functional centrosomes, indicating that all the components needed for the formation of these organelles can be provided maternally. A similar result has been obtained in the artificially activated sea urchin egg (Dirksen 1961) which, as noted above, is a species that normally obtains a centrosome from the sperm. Thus, under certain conditions, at least two species that are generally accepted to have a paternal requirement for provision of a centrosome (Sonnenblick 1950; Schatten et al. 1986) can produce these organelles entirely from maternal products. This result does not rule out the possibility that there is normally a paternal contribution of centrosomes in wild-type development, but it does demonstrate that any such contribution is not essential. The result with GNU embryos is perhaps not surprising, as there are several *Drosophila* species that undergo significant rates of parthenogenesis (Templeton 1983), and a parthenogenetically derived embryo clearly must be derived entirely from maternal components. Although *D. melanogaster* does not normally show significant rates of parthenogenesis, a male sterile mutation, *ms(3)K81* (Fuyama 1984, 1986), has been isolated in which sperm from homozygous males can activate egg development but are unable to participate in syngamy. Some of these activated eggs undergo considerable development, even though all their cells are derived from the female pronucleus. Indeed, Fuyama has established a strain that reproduces gynogenetically when inseminated by *ms(3)K81* males (see discussion of Fuyama 1986), although the nature of the genetic variant in this strain is not yet clear. It is not known whether the centrosomes in these gynogenetic individuals are contributed maternally or by the mutant sperm. The interactions between mutants of this kind, *gnu* and *mh*, will allow us to begin to deduce a network of epistatic interactions between genes involved in the

repression of DNA synthesis in, and the subsequent activation of, the unfertilized egg.

The molecular mechanisms of egg activation are poorly understood (Went 1982). Biochemical studies of amphibian eggs have led to the isolation of factors, such as MPF and cytosolic factor (CSF), that clearly play a part in the metaphase arrest of the unfertilized egg and the subsequent activation of DNA synthesis (Masui and Markert 1971; Smith and Ecker 1971; Meyerhof and Masui 1979; Maller and Krebs 1980; Wu and Gerhart 1980; Miake-Lye et al. 1983). MPF is of particular interest, as it is present in all cell types examined, and its level is found to cycle in unison with the mitotic cell cycle (Gerhart et al. 1984; Newport and Kirschner 1984); it is thus thought to be an important regulatory element of mitosis throughout development, rather than being confined to its role around the time of fertilization. However, the difficulty of purifying these factors means that their precise roles have remained elusive. The defects caused by the *gnu* mutation suggest that the wild-type gene product may participate in the equivalent processes of egg activation in the *Drosophila* egg. The molecular genetic analysis of the *gnu* gene that is now in progress should provide new insights into the mechanisms underlying the onset of DNA synthesis and the consequent initiation of development in the fertilized egg.

Materials and methods

Drosophila stocks and egg collection

Wild-type stocks were from cultures of Oregon-R strain (Lindsley and Grell 1968), maintained continuously in this laboratory. The line of flies carrying the bacterial CAT gene was α T3.1, as described by Davies et al. (1986). The mutation *mh* was the gift of M. Gans, and has been described previously (Gans et al. 1975; Zalokar et al. 1975; Santamaria and Gans 1980). All fly stocks were kept under standard culture conditions (Roberts 1986).

Embryos were collected on petri dishes containing grape juice agar (80 g sucrose, 8 g yeast, 48 ml concentrated grape juice, 25 g agar per liter), supplemented with a yeast paste. These dishes were fitted to the bottoms of one-third pint milk bottles, with their bases removed.

Antibodies

We used the mouse monoclonal antibody Bx63 (Frasch et al. 1986) against *Drosophila* centrosomes and the rat monoclonal YL1/2 (Sera-Lab, UK) (Kilmartin et al. 1982) against tubulin. YL1/2 has been shown to recognize specifically α -tubulin in *Drosophila* (Warn and Warn 1985). Dilutions of between 1 in 5 and 1 in 15 were used for hybridoma culture supernatant, and dilutions of 1 in 500 for ascites fluid, all in Buffer A [15 mM Tris-HCl (pH 7.4), 60 mM KCl, 15 mM NaCl, 1.5 mM spermine, 0.5 mM spermidine]. Rhodamine- and fluorescein-conjugated second antibodies were bought from Jackson Immunoresearch Laboratories, Inc. (USA), and used at 1 in 500 and 1 in 100, respectively.

Embryo fixation and permeabilization

Embryos were fixed and prepared for staining by a technique that has been described previously (Freeman et al. 1986). The embryos were treated with 0.5 μ M taxol, a microtubule-stabilizing toxin, for 30 sec prior to fixation. Under these conditions, taxol is believed not to cause aberrant polymerization of tubulin in *Drosophila* embryos (Karr and Alberts 1986; Freeman et al. 1986).

Antibody staining of embryos

Embryos were stained immunofluorescently and photographed as described previously (Freeman et al. 1986). Antibodies were applied in the order Bx63, goat anti-mouse IgG (H + L), YL1/2, goat anti-rat IgG (H + L).

DNA preparation

DNA was prepared by a modification of a protocol described previously (De Cicco and Glover 1983), using between 50 and 300 embryos in place of a single fly and increasing the volumes by 50%. DNA preparations were treated with proteinase K (100 μ g/ml 45°C, 60 min) and phenol-extracted, before being ethanol-precipitated and resuspended in TE [10 mM Tris-HCl (pH 8.0), 1 mM EDTA].

DNA hybridization probes

The LSP plasmid used as a hybridization probe was a 1.65-kb *Bam*HI fragment from the 5' end of the *Drosophila* LSP 1 α gene (Smith et al. 1981), cloned into pEMBL8(+) (Dente et al. 1983). The CAT plasmid was a 1.64-kb *Bam*HI-*Bgl*II fragment of pSV0CAT (Gorman et al. 1982), which contains the *Escherichia coli* CAT gene, cloned into pEMBL9(+) (Dente et al. 1983).

DNA blotting and hybridization

DNA was immobilized onto nitrocellulose filters using a Schleicher & Schull Slot Blot apparatus, and the manufacturer's instructions were followed. Hybridization was carried out under normal, high-stringency conditions (Maniatis et al. 1982), and the radiolabeled probes were prepared by the oligo-labeling method of Feinberg and Vogelstein (1983, 1984).

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