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Patterns of *engrailed* protein in early *Drosophila* embryos

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Summary

By the onset of gastrulation during nuclear cycle 14 of *Drosophila* embryogenesis, the *engrailed* gene is expressed in fourteen one-cell-wide stripes. Each stripe defines the anlagen of the posterior compartment of a metameric segment. We report here several observations relating to the role and disposition of the *engrailed* protein during the embryonic stages that precede cellularization. We demonstrate that in embryos mutant for the *engrailed* gene, there were characteristic morphological abnormalities as early as the 6th cleavage cycle. In addition, the *engrailed* protein was detected in pre-cycle-9 embryos by Western blot analysis. When localization of *engrailed* protein begins during cycle 14, *engrailed* expression was first present in broad anterior and posterior regions before the fourteen-stripe pattern appeared.

Key words: *engrailed*, *Drosophila*, embryogenesis, antibodies, protein.

Introduction

During the first several hours of *Drosophila* development, nuclei divide and migrate to the embryo periphery, and are separated into individual cells. By this stage (cellular blastoderm), the embryo has established a body plan in which the progenitor cells of the various parts and organs of the subsequent developmental stages are precisely arranged (Lohs-Schardin *et al.* 1979). Although cells of cellular blastoderm embryos are morphologically indistinguishable, different cells express numerous genes in patterns that reflect the segregation of developmental potential within the embryo. For instance, the future posterior compartments of each metameric segment are arranged as single-cell-wide annuli evenly spaced along the anterior–posterior axis of the embryo. The *engrailed* gene is expressed only in these posterior compartment cells (Kornberg *et al.* 1985; Fjose *et al.* 1985).

This pattern of *engrailed* expression is critical to the organization of the early embryo. *engrailed* mutants lacking a wild-type *engrailed* gene do not segment properly. Such mutants develop with fused segments and die late during embryogenesis (Kornberg, 1981; Nüsslein-Volhard & Wieschaus, 1980). In zygotic and maternal effect mutants defective for other segmentation genes, regions in which the *engrailed* gene fails to express normally also fail to form appropriate segments (reviewed in Akam, 1987).

The mechanisms that establish the reiterated pattern of *engrailed* expression are clearly fundamental to the organization of the early embryo. Several kinds of studies indicate that the process that localizes *engrailed* expression is complex. First, studies with the inhibitor of protein synthesis, cycloheximide, indicate that the striped pattern of *engrailed* expression depends upon repression of transcription in the interband regions (Weir *et al.* 1988). Second, *engrailed* expression in the bands requires various other maternal and zygotic segmentation gene products (reviewed in Akam, 1987). Third, the *engrailed* gene is expressed transiently in simpler patterns prior to formation of the fourteen-striped array. As cellularization takes place during nuclear cycle 14, *engrailed* RNA is expressed in patterns with periodicities of four and two segments prior to the final one-segment pattern (Weir & Kornberg, 1985). These intermediate patterns are consistent with the view that genes expressed in larger intervals control the localization of *engrailed* expression.

Our previous studies indicated that the *engrailed* gene is also expressed prior to nuclear cycle 14, as revealed by Northern analysis and by morphological abnormalities characteristic of *engrailed* mutant embryos older than nuclear cycle 8 (Karr *et al.* 1985). In this study, we confirm these observations and extend, to at least the 6th nuclear cycle, the precellular role of the *engrailed* gene. Furthermore, we used sensitive monoclonal antibody probes directed against the *engrailed* protein to resolve the sequence of patterns through which *engrailed* expression evolves during early nuclear cycle 14. We demonstrate that prior to the formation of narrow stripes, the *engrailed* gene is expressed in a series of novel patterns that precede those previously observed (Weir & Kornberg, 1985; DiNardo *et al.* 1985).

Materials and methods

Fly culture

Flies were raised under standard conditions. For either cytological or biochemical studies, *engrailed* mutants (Kornberg, 1981) were outcrossed to Oregon R wild type and the heterozygous progeny were crossed *inter se*. en^{LA4} is an EMS-induced allele with a translation stop at codon 422 (E. Gustavson and Kornberg, unpublished). en^{SFX31} is an X-ray-induced deletion of the *engrailed* locus and surrounding chromosomal region.

Morphological examination of early embryos

Embryos were collected under water with a paint brush, transferred by pipette to a wire mesh screen and washed extensively before the chorion membranes were removed by immersion in a dilute solution of commercial bleach (active ingredient 2·6% sodium hypochlorite). Embryos were washed extensively with a Triton X-100 solution (containing 0·03% Triton X-100 and 0·7% NaCl) to remove the bleach and were subsequently transferred in the same solution to Petri dishes for microscopic examination.

Living dechorionated embryos from either wild-type or mutant parents were examined with a dissecting microscope at 50× magnification and their developmental age determined as follows: pre-cycle 9, by the absence of pole cell protrusions at the posterior end; cycles 10–14, by the appearance over the entire surface of somatic buds; cellular blastoderm, by invaginating membrane furrows that synchronously envelope each blastoderm nucleus; gastrulae, by the obvious and regular movements of the pole cells as they migrate up onto the dorsal midline of the embryo, and by invaginating cells of the ventral and cephalic furrows.

In control experiments, embryos from each of these stages were selected, fixed in formaldehyde and stained with the DNA-specific fluorochrome DAPI (4′,6′-diamidino-2-phenylindole) as described below. Their developmental age could be unambiguously determined by counting nuclei present in each embryo. Embryos selected as described were staged with greater than 99% accuracy for pre-cycle 9 and gastrulating embryos, and with greater than 95% accuracy for the intermediate periods.

Fixed embryo preparations

Embryos were fixed by either of two methods. The formaldehyde/methanol fixation method initially described by Mitchison & Sedat (1983) and subsequently modified by Karr *et al.* (1985) was employed with further modifications. 100–200 hand-selected embryos were transferred to a 50 ml screw-top tube that contained 15 ml of 0·1 M-Pipes (pH 6·9 with KOH); 1 mM-EGTA, 1 mM-$MgSO_4$. 5 ml of reagent grade heptane was immediately added along with 2·0 ml of a 37% solution of formaldehyde, and the two-phase solution was shaken vigorously for 25–30 min. The outer vitelline membrane was removed by transferring the fixed embryos from the heptane/buffer interface to a solution containing a 1:1 mixture of methanol and heptane (the methanol solution was 90% methanol and 10% H_2O containing 0·05 M-EGTA) that had been cooled to −70°C with dry ice. This suspension was vigorously shaken for 10 min and was rapidly warmed under a stream of hot tap water until the majority of embryos had dropped to the bottom of the flask. An alternative method using warm instead of cold methanol was employed with similar results. The few embryos that were not devitellinized by this process remained at the interface and were discarded. Embryos were removed with a pipette to a test tube, washed three times with the methanol solution and then hydrated directly into PBS in preparation for immunohistochemical and/or fluorescent staining as detailed below.

Immunohistochemistry and microscopy

To examine embryonic nuclei, embryos were treated with DAPI for 5 min in PBT (PBS containing 0·1% Triton X-100), rinsed in PBT, mounted and observed as described below. For reaction with anti-*engrailed* antibodies, fixed and devitellinized embryos were treated first in PBS containing 3% hydrogen peroxide for 15 min to inhibit endogenous peroxidase activity. Embryos were washed free of peroxide with three changes of PBT and then transferred into a solution of PBS containing 10% normal goat serum, and treated with anti-*engrailed* monoclonal antibody (Gay *et al.* 1988) that had been previously purified over protein A-sepharose (Pharmacia, as per instructions). Typically, 100–200 embryos were incubated with approximately 1–5 $\mu g\,ml^{-1}$ antibody in 0·5 ml reaction volumes for 1–4 h at room temperature. Embryos were rinsed three times in PBT and washed in PBT for 1–2 h at room temperature. Following this wash, embryos were incubated for 1 h in 0·5 ml PBS containing 10% normal goat serum supplemented with 1–3 μg of biotinylated horse anti-mouse secondary antibody (Vector Laboratory). Embryos were rinsed and washed as before, and were treated with 1 μg streptavidin–horse radish peroxidase (Bethesda Research Laboratories) in 0·5 ml of PBT (all buffers were azide-free). Finally, embryos were rinsed in PBT and peroxidase reaction product was developed by the addition of diaminobenzidine (DAB) to 0·2 $mg\,ml^{-1}$ in the presence of 0·05% hydrogen peroxide. The reaction was observed in the dissecting microscope until the desired intensity was obtained (usually about 3–5 min at room temperature) and was terminated by washing in PBT containing 0·05% sodium azide.

Microscopy

For light microscopic examination, embryos were dehydrated in ethanol, transferred to xylene and transferred to a 1:1 mixture of xylene and Permount. Embryos in Permount were mounted under glass coverslips on microscope slides and warmed to 55°C for at least 12 h prior to observation. Embryos stained with DAPI were prepared by pipetting embryos in PBT onto slides, gently aspirating away excess buffer and adding a drop of aqueous mounting media (Fluoromount G, Southern Biotechnologies). All embryos were observed and photographed using a Zeiss Universal microscope equipped with bright-field, Nomarski DIC and epifluorescence optics using Zeiss 16X or 25X Plan-Neofluor lenses. Images were recorded on Kodak Technical Pan 2415 film and developed using Kodak HC-110 developer at dilution E.

Immunoblot analyses

Embryos were staged, homogenized in SDS sample buffer, boiled for 5 min and fractionated by electrophoresis. A control lane containing 1 mg of E. coli *engrailed* protein (the complete coding portion of cDNA c-2.4 (Poole *et al.* 1985) was expressed in the phage T7 system of Studier (Dunn & Studier, 1986); Rosen & Kornberg, unpublished). Proteins were transferred to nitrocellulose by transverse electrophoresis (16 h at 0·2 A), and were prepared for analysis as follows. All procedures were performed at room temperature. Blots were washed three times with PBT followed by treatment with PBT containing 10% normal goat serum for at least 30 min. In the PBT/normal goat serum solution, blots were probed for 1 h with 10–20 $\mu g\,ml^{-1}$ of an anti-*engrailed* monoclonal antibody. Blots were rinsed for more than one hour with numerous changes of PBT, treated with a solution of

PBT/normal goat serum containing ^{125}I-anti-mouse IgG (Amersham) for 1 h, exhaustively rinsed and washed in PBT, dried and exposed to X-ray film (Kodak XR with intensifying screen) for 2 days at −70°C.

Results

engrailed *functions in cleavage-stage embryos*

Previous studies demonstrated that the *engrailed* gene functions from at least nuclear cycle 9 onwards, when nuclei first reach the periphery of the embryo. Cycle 9 *engrailed* mutant embryos are morphologically abnormal. They have uneven spacing of their somatic nuclei and lateral displacement of their pole cells (Karr *et al.* 1985). These embryos die at about 18 h after fertilization with pair-wise fusions of their segments.

To determine whether the *engrailed* gene functions earlier in development, during the cleavage stages prior to cycle 9, embryos from crosses of heterozygous *engrailed* parents were examined for morphological abnormalities. If the *engrailed* gene has a zygotic function during the cleavage stages, one quarter of the embryos might be expected to be morphologically abnormal. Unfortunately, morphological abnormalities were not visible in live embryos due to their optical opacity. Therefore, embryos were fixed and stained for fluorescence microscopy with a DNA-specific fluorochrome, DAPI. These embryos were easily staged and scored, since the early nuclear divisions are virtually synchronous and the number of nuclei and their mitotic stages are readily visible (e.g. nuclear cycle 6 embryos have 32 nuclei).

Although the cleavage nuclear divisions were remarkably uniform in wild-type embryos, deviations from mitotic synchrony were observed among the progeny of heterozygous *engrailed* parents (Fig. 1A,B). Of 258 wild-type embryos examined, all nuclei in 255 individuals (97%) were in the same stage of the mitotic cycle. In contrast, 18–26% of embryos from *engrailed* heterozygous parents were clearly asynchronous ($n = 376$; Fig. 1; Table 1). Most of these abnormal embryos were in nuclear cycles 6–9, and embryos from these crosses that were younger than cycle 6 were not significantly different from wild type. We conclude that the *engrailed* gene has a zygotic function during the cleavage stages prior to formation of the syncytial blastoderm, and is activated at or before nuclear cycle 6 when the embryo has 32 nuclei.

Table 1. *Genetic control of mitotic synchrony*

engrailed alleles ♂	*engrailed* alleles ♀	% mitotic asynchrony: Nuclear cycles 1–9	1–5	6–9	Σ Scored
WT*	WT	3	3	3	(258)
LA4	SF31	18	3	26	(106)
LA4	LA4	17	5	25	(101)
SF31	SF31	22	6	20	(169)

*Oregon R.

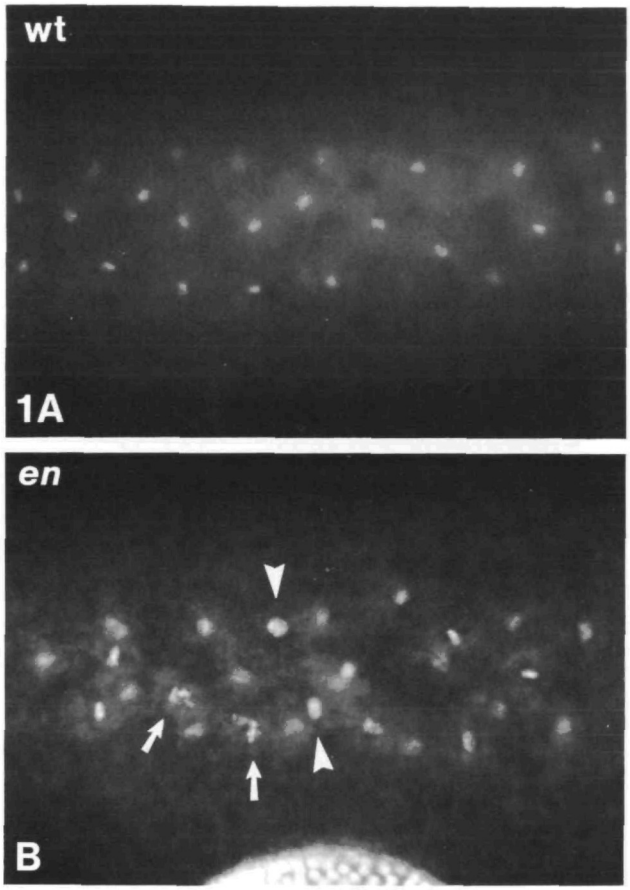

Fig. 1. Nuclear synchrony in wild-type and *engrailed* cleavage-staged embryos. Wild-type (A) and *engrailed* mutant (B) embryos were fixed and stained with a DNA-specific dye, DAPI, as described in Methods. Embryos were examined using epifluorescence illumination and the degree of mitotic synchrony compared. (A) In this typical wild-type nuclear cycle 6 embryo, the nuclei are approximately synchronous. (B) In this mutant embryo of about the same age, the nuclei are out of synchrony; the arrowheads indicate nuclei in interphase as judged by the degree of chromosome condensation, and the arrows point to more condensed nuclei indicative of metaphase.

engrailed *protein is present in precellular embryos*

Previously, *engrailed* RNA transcripts were shown to be present in precellular embryos (Karr *et al.* 1985). To directly test for the presence of *engrailed* protein in cleavage and syncytial blastoderm-stage embryos, monoclonal anti-*engrailed* antibodies were used to probe immunoblots of whole embryo extracts. Embryos were staged under a dissecting microscope into three categories, cleavage stage (cycles 1–9), syncytial and cellular blastoderm (cycles 10–14), and gastrulae, and were extracted in SDS–PAGE sample buffer. After electrophoretic fractionation and transfer to nitrocellulose membranes, *engrailed* protein was monitored with anti-*engrailed* antibodies and ^{125}I-antimouse IgG. In each of the three staged populations, a protein species was detected which migrated in a manner indistinguishable from *engrailed* protein that had been produced in

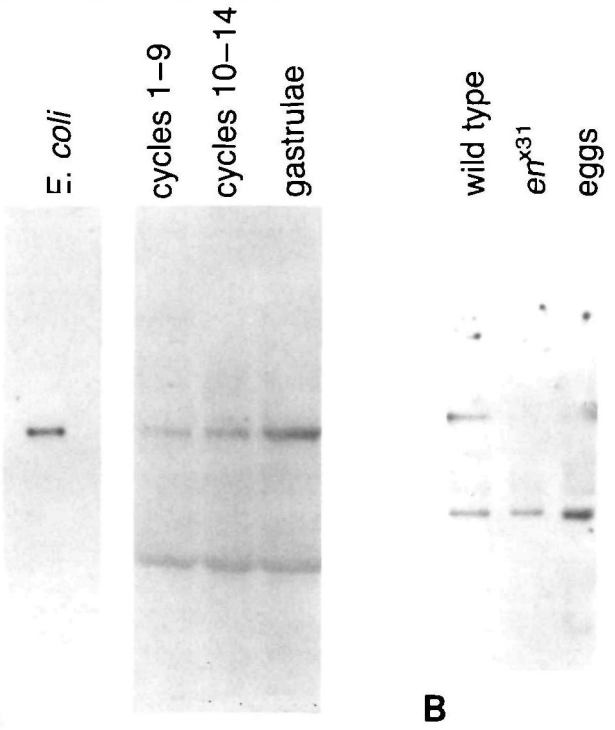

Fig. 2. Immunoblot analyses in *Drosophila* embryo extracts. All lanes (except the first and last lanes) are autoradiographs of immunoblots probed with an anti-*engrailed* mouse monoclonal antibody and an iodinated IgG secondary antibody. (A) The left lane shows the electrophoretic mobility of *engrailed* protein produced in *E. coli* and is followed by 3 lanes with extracts of embryos whose age is indicated above each lane. (B) Comparison of the immunoreactivity of the anti-*engrailed* antibody in wild-type embryos, homozygous en^{SFX31} deficiency embryos and unfertilized eggs. Mutant embryos were identified by their gastrulation phenotype (Karr *et al.* 1985). 100 embryos per lane.

E. coli (Fig. 2). Although a second band of protein bound the antibody in these *Drosophila* preparations, this lower molecular weight species migrated in the region of the abundant yolk proteins, was recognized directly by anti-mouse IgG, and was not detected when a streptavidin–biotin detection system was used (not shown).

To establish whether the monoclonal antibody specifically recognized the *engrailed* protein, the relative affinity of the antibody for extracts of wild-type and *engrailed* mutant embryos was compared. Immunoblot analysis was performed on (i) cellular blastoderm embryos from wild-type parents, (ii) mutant cellular blastoderm embryos selected from parents heterozygous for a deletion of the *engrailed* gene, and (iii) unfertilized wild-type eggs. Protein reacting with the anti-*engrailed* antibody was observed only in the wild-type cellular blastoderm embryos. No *engrailed* protein was detected in either the mutant or unfertilized embryos (Fig. 2B). We conclude that *engrailed* protein is synthesized in cleavage and syncytial blastoderm stages as well as during gastrulation.

Relative estimates of *engrailed* protein abundance were obtained from the immunoblot autoradiographs.

Table 2. *Relative abundance of* engrailed *protein*

Nuclear cycle	*engrailed* protein per embryo*	Nuclei (relative no. per embryo)†
Pre-9	1	1
10–14	8	16
14+	20	60

* Estimated from densitometric scan of autoradiograph in Fig. 2, correcting for differences in number of embryos per lane.

† Number of nuclei in the Pre-9 sample, taken as standard (approximately 100 nuclei per embryo), was estimated directly from nuclear counts of fixed and stained embryos. Other values are from published estimates.

Amounts of *engrailed* protein increased approximately 20-fold from the cleavage- to the gastrula-stage preparations (Table 2). This compares to the approximately 60-fold difference in number of nuclei in the preparations, although it is noteworthy that less than one-third of the cells of a gastrula express *engrailed* (see Fig. 3).

Spatial distribution of engrailed *protein during cellularization*

Previous studies probing *engrailed* RNA by *in situ* hybridization and *engrailed* protein with antibodies have indicated that localized synthesis of *engrailed* RNA and protein begins during nuclear cycle 14 (Weir & Kornberg, 1985; DiNardo *et al.* 1985). A single stripe of expressing cells (stripe 2) appears at about 65 % egg length (EL; measured from the posterior pole) early in this cycle, and thirteen additional stripes are subsequently added. (Stripes 1–14 are in parasegments 1–14, respectively.) A generally anterior-to-posterior order characterizes the appearance of these stripes, although 'precocious' expression of certain more posterior stripes occurs. Thus, expression evolves through a series of intermediate patterns from the one with a single stripe 2 to one with: stripes 2 and 8; stripes 2, 4, 8, 12; stripes 2, 4, 6, 8, 10, 12, 14; and stripes 1, 2, 3, 4 ... (also see Fig. 4). The *engrailed* protein in the stripes is localized to the nuclei of the expressing cells.

To relate these patterns of *engrailed* expression to the demonstrated presence of *engrailed* RNA and protein in earlier developmental stages, a histological study of the disposition of *engrailed* protein was undertaken with the monoclonal anti-*engrailed* antibody. Low level and uniform binding of the anti-*engrailed* antibody to precellular blastoderm embryos was observed (not shown). Nonuniformity in patterns of *engrailed* expression was first detected early in cycle 14 (Fig. 3; Fig. 4A–C), as nuclei began to elongate and membrane furrows invaginated between the nuclei (Fig. 4A–C). At this time, expression in the middle third of the embryo (35–60 % EL) lessened significantly relative to the anterior (60–100 % EL) and posterior (0–35 % EL) thirds. Later in cycle 14 (Fig. 4D), expression in the anterior 85–100 % EL diminished, and the broad region of expression between 60–85 % EL split into two stripes (Fig. 3D). Expression in the posteriormost 0–15 % EL started to reduce in intensity at this stage.

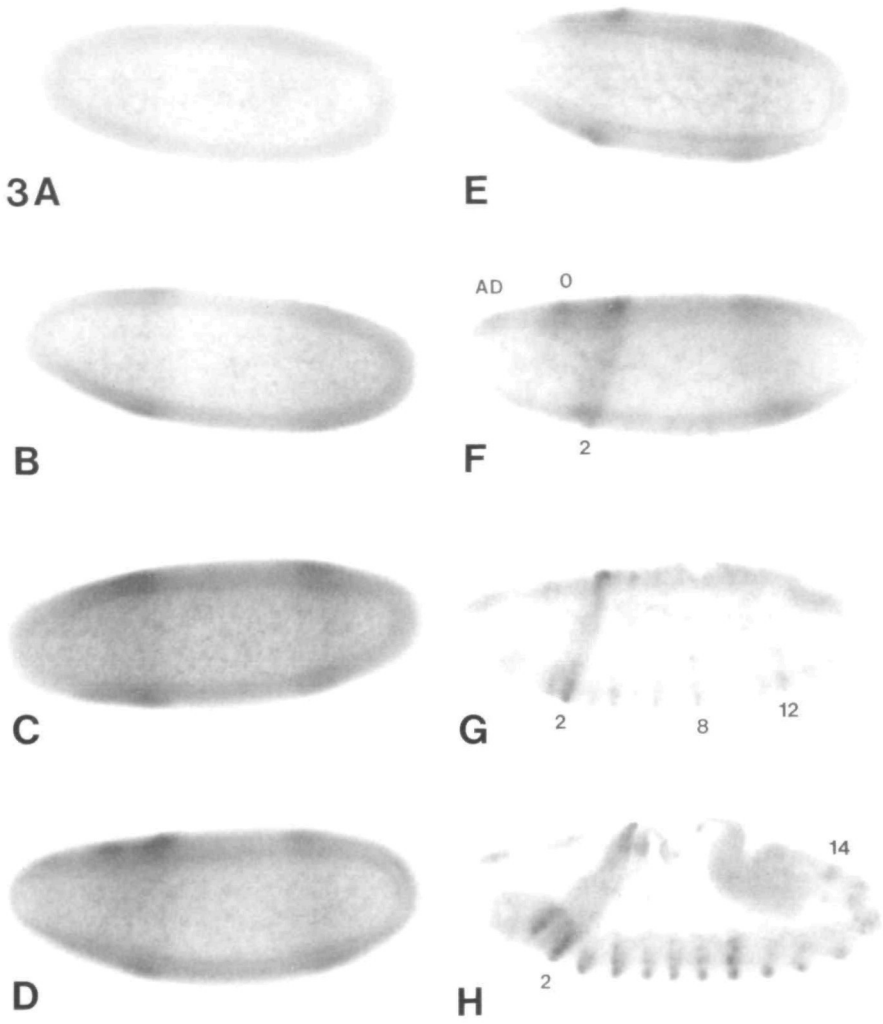

Fig. 3. Localization of *engrailed* protein in blastoderm embryos. (A–H) Bright-field images of intermediate patterns of *engrailed* expression: (A) nuclear cycle 13; (B–H) progressively older embryos in cycle 14 (see Fig. 4). Over 400 embryos were photographed and analysed.

As cycle 14 proceeded, more areas of localized *engrailed* protein were detectable. In the anterior dorsal part of the embryo centred at 90% EL, a region of expression was evident (Fig. 3D–G). Additional stripes also appeared in the middle of the embryo (Fig. 3E,F). The axial positions and relative strengths of bands (Fig. 3G) allows assignment of identities to stripes in accordance with previous descriptions of *engrailed* stripe formation (Weir & Kornberg, 1985). Stripe 2 can be identified with the prominent broad band centred at 65% EL (cf. Fig. 3C–G). The broad band centred at 35% EL focuses in the region of stripes 11–13 (cf. Fig. 3F and G). The broad band at 80% EL focussed into a band immediately anterior of stripe 1. We refer to this as stripe 0. Expression in this band and in the anterior dorsal stripe at 90% EL remained relatively low until later during germband elongation when it became more prominent. The location of stripe 0 in germband elongated embryos suggests that it may be the posterior compartment of the antennal segment; expression in the anterior dorsal band appears to contribute to later labial expression. These observations are summarized in Fig. 5.

Discussion

The engrailed *protein*

Previous studies defined the *engrailed* transcription unit (Drees *et al.* 1987), the sequence of its most abundant transcript (Poole *et al.* 1985), its patterns of expression (Kornberg *et al.* 1985; Fjose *et al.* 1985; Weir & Kornberg, 1985), and the localized appearance of *engrailed* protein in the nuclei of posterior compartment cells in gastrulating embryos (DiNardo *et al.* 1985). The analysis presented here used a monoclonal antibody directed against the *engrailed* protein for histological and molecular studies. We show that the *engrailed* protein is present in precellular embryos, substantiating our previous observation that the *engrailed* locus is expressed prior to cellularization. We also compared protein isolated from embryos to protein synthesized in *E. coli* whose sequence corresponded precisely to the coding portion of the *engrailed* transcription unit. No difference in mobility of protein isolated from embryos or bacteria was detected. We conclude that the *engrailed* protein exists in the cell without substantial proteolytic processing and that the

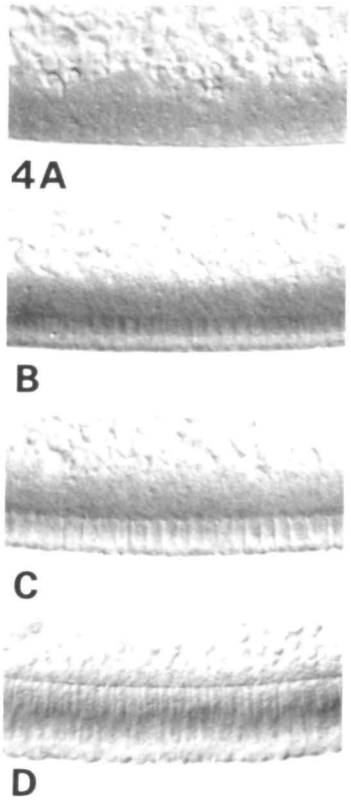

Fig. 4. Morphology of stained embryos. (A–D) Nomarski differential interference contrast images of nuclei in embryos A–D of Fig. 3. Nuclear densities indicate that A is in cycle 13 and B–D are in cycle 14. During cycle 14, the nuclei elongate and become separated by cell membranes.

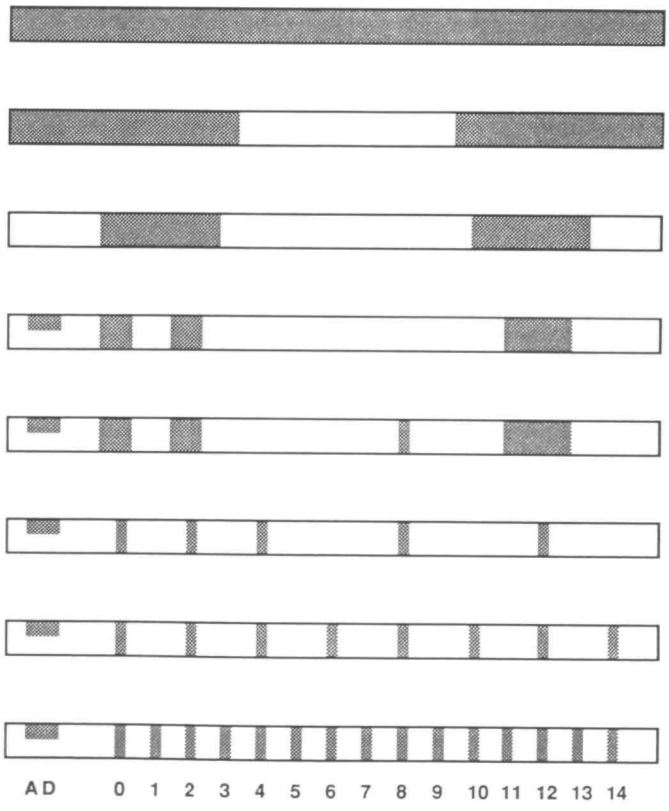

Fig. 5. Schematic summary of *engrailed* protein patterns. Detectable expression (shaded) changes from uniform to striped during nuclear cycle 14. Regions of early expression become narrower and more pronounced with time.

entire potential protein coding sequence of the cDNA that was analysed (Poole *et al.* 1985) is utilized *in vivo*.

Early engrailed *function*

There is an extensive collection of *Drosophila* mutants whose phenotypes portray a logical pathway of developmental decisions for establishing the cellular blastoderm body plan (reviewed in Akam, 1987). Among these are: maternal-effect mutants which affect the global organization of the embryo and alter overall polarity and spacing of metameres; gap mutants which affect contiguous blocks of segments and eliminate portions of the body plan; pair-rule mutants which affect alternate segments and eliminate either odd- or even-numbered segments; segment-polarity mutants which affect patterns in each segment; and homeotic mutants which change the developmental pathway adopted by individual segments or parasegments. These phenotypes suggest a sequential role for these different genes as the developmental potential of embryo constituents become progressively restricted. Indeed, temporal control of expression of many of these genes has been shown to correlate well with such a pathway: most maternal-effect genes are expressed exclusively during oogenesis; gap genes are first expressed during the first syncytial blastoderm cycles; pair-rule genes exhibit localized expression early in cycle 14; segment-polarity genes are localized later in cycle 14; and the localized and restricted expression of homeotic genes is established in the early gastrula.

It is therefore surprising that some of these genes function prior to the stages when their expression is localized to discrete regions of the embryo. It is striking and was unexpected that morphological abnormalities among the progeny of *engrailed* heterozygous parents could be detected as early as the 6th nuclear cycle. Cycle 6 embryos are less than 90 min postfertilization and have only 32 nuclei. Yet our molecular and genetic studies indicate that the *engrailed* protein of these embryos is not maternally derived. Neither *engrailed* protein (Fig. 2) nor mRNA (Karr *et al.* 1985) is present in unfertilized eggs. If the mutant phenotype is a consequence of the lack of *engrailed* function, how soon after fertilization is the *engrailed* gene expressed? We do not have direct evidence for *engrailed* protein synthesis during the first several nuclear divisions, since our antibody probes were not sufficiently sensitive to detect *engrailed* protein in whole-mount preparations of cleavage-stage embryos. However, since several nuclear cycles may elapse before the cycle 6 phenotype can be manifested, it is likely that expression of the *engrailed* gene initiates during the first several cycles.

During the 20–30 min that follows fertilization and precedes the first mitotic division of the *Drosophila* zygote, meiotic divisions generate the female pronucleus and chromosomes replicate. For approximately the next 90 min, the nuclei divide synchronously with a cycle time of about 12 min. It is not known if the embryo is transcriptionally competent at these stages, although the zygotic nucleus of the beetle, *Leptinotarsa* (Coleoptera), incorporates ribonucleotides (Schenkel &

Schnetter, 1979). Similar studies utilizing autoradiography of embryos injected with labelled substrate have not been carried out in *Drosophila*. Edgar & Schubiger (1986) isolated RNA from injected *Drosophila* embryos and detected zygotic RNA synthesis prior to nuclear cycle 10, but attributed this activity to mitochondria. At a very low frequency, however, McKnight & Miller (1976) did observe small transcription complexes in the chromosomes of cleavage-stage embryos. Our more recent studies using the sensitive polymerase chain reaction technique (Saiki *et al.* 1985), confirm the presence of *engrailed* transcripts in cleavage-stage embryos (Maschat, F. and Kornberg, T., unpublished).

Synthesis of *engrailed* protein and the requirement for *engrailed* function in cleavage-stage embryos underscores both the existence of zygotic expression during this early period of *Drosophila* development and its importance. What is its role? Since mutant phenotypes of *engrailed* and other genes (e.g. *even-skipped*, Ali and Kornberg, unpublished) include loss of mitotic synchrony and uneven spacing of nuclei, it is likely that these genes have a role in regulating nuclear division in the early organization of the *Drosophila* embryo.

Patterned expression of the engrailed *gene*

Histological studies resolved a remarkably complex series of patterns of *engrailed* expression in the pregastrula embryo. The precise zebra-like pattern of *engrailed* expression in the early gastrula – fourteen rings of expressing cells, each 1–2 cells wide and separated by 2–3 non-expressing cells – evolves from earlier patterns which encompass larger areas of the embryo.

In cleavage-stage and syncytial blastoderm embryos, *engrailed* protein is expressed, but at levels insufficient for histological analysis with available antibody probes. Between the beginning of nuclear cycle 14 and the time of gastrulation, patterns of *engrailed* protein changed rapidly. Initially, three broad and rather diffuse bands of protein appeared and grew in intensity. Later in the nuclear cycle as gastrulation commenced, additional well-focussed and intense bands of protein joined the increasingly complex pattern. Note that it is not until mid-cycle 14, when the embryos are cellularizing and the syncytial divisions have ceased, that *engrailed* expression is localized and its protein concentrated in nuclei. Such nuclear localization characterizes all subsequent patterns of the *engrailed* protein in the embryo (DiNardo *et al.* 1985).

Many studies have described how mutations in other segmentation genes influence the zebra-striped pattern of *engrailed* expression in the early gastrula. From the observed changes in *engrailed* expression, several genes have been proposed to be either positive or negative regulators of the *engrailed* gene. Given that these other genes are themselves expressed in zebra-striped patterns which partially overlap with the *engrailed*-expressing cells, a role for these genes in regulating the *engrailed* gastrula patterns is certainly attractive. In view of the sequence of patterns of *engrailed* expression revealed in this study, however, it will also be important to determine how the spatial domains of expression of the different genes correlate during the earlier periods of rapid pattern evolution in order to understand fully how these various patterns form.

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