Association of the *Drosophila melanogaster engrailed* protein with specific soluble nuclear protein complexes

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The *Drosophila engrailed* protein which contains a homeobox domain and specific DNA binding activity is believed to function in the regulation of gene expression during embryogenesis. Here we show that the *engrailed* protein interacts stably with specific complexes of soluble nuclear proteins when expressed artificially in a cell line and in the developing embryo. The *engrailed* complexes have molecular masses between 10^7 and 10^8 which suggests they contain a polymeric protein component. The complex is able to bind reversibly to DNA and a definitive purification shows it to be constituted of 12 distinct protein species, two of which are predominant. Purified, bacterially produced *engrailed* protein can be reconstituted with both culture cell and embryo nuclear protein fractions to form complexes of the same and related composition respectively. On the basis of these results we propose that protein – protein interactions as well as DNA binding are important for correct *engrailed* protein function *in vivo*.
Key words: Drosophila melanogaster/engrailed/gene expression

Introduction

The development of the *Drosophila melanogaster* embryo commences with very rapid, synchronous divisions of the zygotic nucleus. At nuclear division cycle 10 most of the nuclei have moved to the periphery of the egg to form the syncytial blastoderm and the prospective germ cells form at the posterior pole. By the end of nuclear division cycle 14 plasma membranes enclose the syncytial nuclei to form the cellular blastoderm and shortly thereafter the morphogenetic cellular movements that characterize the process of gastrulation commence (Karr and Alberts, 1986).

An important developmental process that occurs at about the time the cellular blastoderm forms is the progressive subdivision of the embryo to form the compartments and segments which will differentiate to form the characteristic structures of the adult insect. The *engrailed* gene is essential for this process of subdivision and embryonic lethal mutants lacking normal *engrailed* gene activity show severe disruption in the formation of the normal segmental pattern of the embryo (Kornberg, 1981).

Expression of the *engrailed* gene can first be detected at nuclear division cycle 10 although phenotypic effects are

manifest in precellular, *engrailed* mutant embryos (Karr et al., 1985). By nuclear cycle 14, *engrailed* transcripts are found to be localized into 14 distinct 2-cell wide stripes spaced along the anterior – posterior axis of the embryo and these stripes of *engrailed* expression define the anlagen of the posterior compartment of each segment of the adult insect (Fjose et al., 1985; Kornberg et al., 1985). The *engrailed* protein is detected in the same iterative pattern of 14 stripes and is seen to become localized in the cell nucleus (Di Nardo et al., 1985). The primary structure of the *engrailed* protein is unusual with stretches of polyglutamine and polyalanine and towards the C-terminal end a highly conserved domain called the homeobox, which is found in many other *Drosophila* gene products involved in the process of segmentation (Poole et al., 1985).

Taken together with genetic evidence, the nuclear localization of the *engrailed* protein supports the hypothesis that *engrailed* is a 'selector' gene that functions to regulate the expression of a subset of 'realisator' genes which directly determine the differentiated cellular state (Garcia-Bellido and Santamaria, 1972). In this regard, the *engrailed* protein is able to mediate site specific DNA binding *in vitro* and this activity appears to reside in the C-terminal section of the molecule that includes the homeobox domain (Desplan et al., 1985). However, such sites of interaction occur very frequently in *Drosophila* DNA (every 1 – 2 kb) and are also found in unrelated molecules such as λDNA. Thus it seems likely that many such sites of *engrailed* protein – DNA binding are of no functional significance and it may be that genuine regulatory *engrailed* protein – DNA binding involves synergistic interactions with other nuclear components not present in the *in vitro* DNA binding reaction.

In this paper we report that the *engrailed* protein, overproduced in a cell-culture line and in the developing embryo can associate stably with specific, soluble nuclear protein complexes. We propose that protein – protein interactions as well as site specific DNA binding are important for the correct function of the *engrailed* protein in the regulation of gene expression *in vivo*.

Results

Overexpression and nuclear localization of engrailed protein in a transformed Schneider 2-cell line HS – EN

Cell line HS – EN was made by transforming the Schneider 2-cell line with a plasmid construct which places the complete *engrailed* cDNA sequence under the control of the *Drosophila* heat shock promoter, HSP70 (Rio and Rubin, 1985; S.Poole, unpublished results). HS – EN cells do not express detectable levels of *engrailed* protein when grown at 25°C but when incubated at 37°C for 30 min, a high level of *engrailed* expression is induced and *engrailed* protein continues to accumulate for at least 3 h after the cells are returned to 25°C. The *engrailed* protein in HS – EN cells can be detected by immunofluorescence microscopy and is
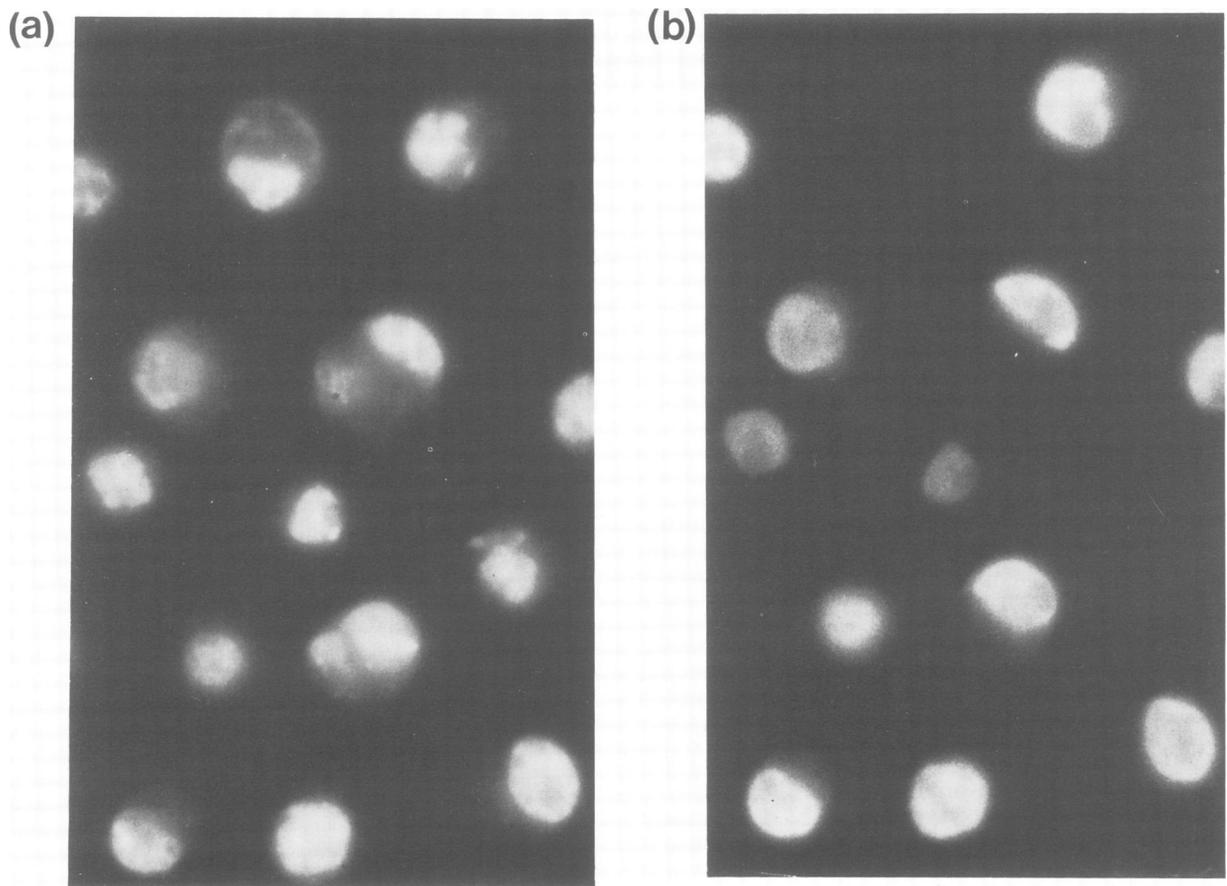
Fig. 1. A suspension culture of HS−EN cells was subjected to heat shock (37°C for 30 min) and allowed to recover at 25°C for 2 h. Samples were prepared for immunofluorescent microscopy as described in Materials and methods. (a) Fluorescent staining pattern of DNA specific dye DAPI. (b) Staining pattern of the same cells with EN2 monoclonal antibody.

seen to be localized almost exclusively in the nucleus (Figure 1a,b). Thus, whilst *engrailed* protein is overexpressed under the control of the heat shock promoter it remains functional by the criterion of nuclear localization. After 4 h of recovery from heat shock, the immunofluorescent staining of nuclear *engrailed* protein appears punctate (result not shown), a phenomenon which has also been observed with the homeo-box protein *caudal*, and was attributed to binding to AT-rich satellite DNA sequences (MacDonald and Struhl, 1986).

Gel filtration chromatography of soluble nuclear protein extracts prepared from induced HS − EN cells reveals that the engrailed protein can form large molecular mass complexes

As a first step of purification, soluble nuclear protein extract prepared from induced HS−EN cells was fractionated by gel filtration in a column of Sepharose 6B-C-L. Samples of the fractions which eluted from the column were analysed for the presence of *engrailed* protein by the Western blot technique. As shown in Figure 2 *engrailed* protein is detected in two distinct peaks, one of which elutes at approximately the position expected for a monomeric protein species of M_r 60 kd. By contrast the second peak elutes in the exclusion volume of the column which indicates a size in excess of M_r 2×10^6. In order to determine whether the *engrailed* protein in the exclusion volume of the column is associated with nucleic acids, the nuclear extract was treated with ribonuclease (0.5 mg/ml) and deoxyribonuclease

(0.25 mg/ml). This treatment removes all of the material which absorbs at 260 nm from the void volume fractions of the Sepharose column but the elution profile of *engrailed* protein remains unaltered as does its size distribution measured by analytical glycerol gradient ultracentrifugation (see Figure 5). In addition ribonuclease resistant RNA species in the Sepharose void volume fractions can be detected by 3'-end labelling with pCp but such molecules are not purified by immunoprecipitation with anti-*engrailed* antibodies. We have also found that *engrailed* is not dissociated by treatment with 1 M KCl, 1 M 2-mercapto-ethanol, 0.1% Triton X-100 and 0.4% NP40, 0.1% SDS but is partially denatured by treatment with 2 M urea and almost completely by 4 M urea (see Figure 5c and d).

The engrailed protein in purified Drosophila embryo nuclear protein extracts can also form high molecular mass complexes

In order to determine whether *engrailed* protein complexes are formed in the developing embryo and thus are not artefacts of heat shock or over expression in HS−EN cells, the size distribution of *engrailed* protein in soluble nuclear protein extracts of 2- to 12-h-old embryos was examined. The concentration of *engrailed* protein in embryo nuclei is low and cannot readily be detected by Western blot analysis of crude embryo extracts. Accordingly, the nuclear protein was first purified 10-fold by DEAE ion-exchange chromato-graphy (see legend to Figure 3). The purified nuclear protein
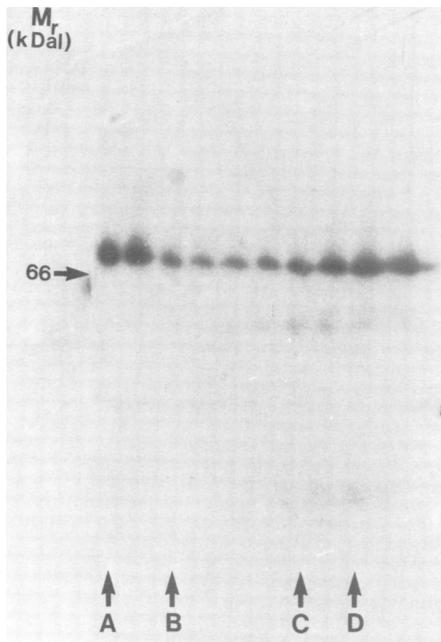
Fig. 2. Size distribution of *engrailed* protein in soluble nuclear protein extracts of induced HS−EN cells. HS−EN nuclear protein extract (12 mg/ml, 0.25 ml) was applied to a column of Sepharose 6B C-L (25 ml, 50 cm long) equilibrated with buffer C plus 0.6-M KCl. Fractions (0.9 ml) were collected and 20-μl samples were subjected to SDS−PAGE (9%). Protein in the gel was transferred to nitrocellulose paper by Western blot and *engrailed* protein detected with monoclonal antibody EN2. The column was calibrated with marker proteins and the position at which each eluted is indicated as follows. **A**, Dextran blue 2000 (M_r 2 × 10^6); **B**, thyroglobulin (M_r 6.7 × 10^5); **C**, gamma globulin (M_r 158 000); and **D**, ovalbumin (M_r 44 000).

Fig. 3. Size distribution of *engrailed* protein in purified nuclear protein extract of early embryo. Nuclear protein extract of 2−12-h embryos (1.5 ml, 10 mg/ml) was applied to a column of DEAE Sepharose 6B C-L (2.5 ml) which had been equilibrated in buffer C. The column was eluted with gradient of KCl (0.1−0.7 M; 7.5 ml). Fractions with KCl concentrations between 0.25 and 0.4 M were pooled and applied to a column of Sepharose 6B C-L. Fractions which eluted from this column were analysed for the presence of *engrailed* protein as described in the legend to Figure 2. M, 1 μg of induced HS−EN cell nuclear protein extract.

was then chromatographed on a column of Sepharose 6B C-L and the fractions were analysed for the presence of *engrailed* protein by Western blotting. As can be seen in Figure 3 about 25% of the embryo *engrailed* protein is detected in the exclusion volume fractions and about 10% at a position corresponding to M_r ~500 000.

This experiment shows that association of *engrailed* with other nuclear proteins is not confined to HS−EN cells but is also observed in the developing embryo.

The engrailed complex accumulates to a steady state level in induced HS−EN cells

The accumulation of *en* complex and low M_r forms was examined *in vivo*. A culture of HS−EN cells was induced to express *engrailed* and samples were taken at five times after induction. Nuclear protein extracts were prepared from the samples and chromatographed in a column of Sepharose 6B C-L. The *engrailed* protein in the complex and low M_r peaks was quantitated for each timepoint using a solid phase radioimmunoassay (see Materials and methods and legend to Figure 4). As shown in Figure 4 the low M_r form of *engrailed* protein continues to accumulate for at least 180 min after induction, but by contrast the complex form accumulates for 60−90 min and then reaches a steady-state level.

This experiment shows that the *engrailed* protein interacts *in vivo* with a specific and saturable nuclear component in HS−EN cells.
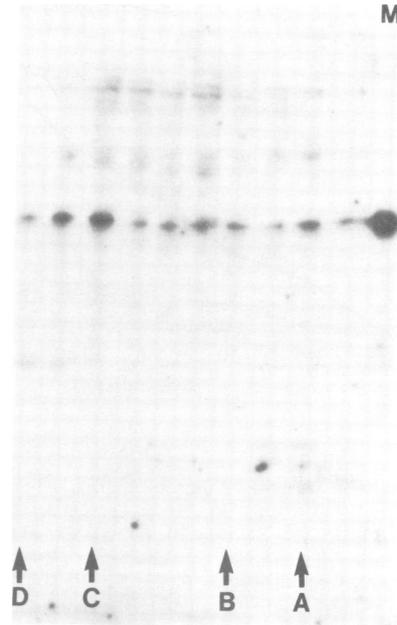
Determination of the molecular mass of the engrailed complex by velocity gradient ultra centrifugation

As the *engrailed* complex elutes in the exclusion volume of the Sepharose 6B gel filtration column, this method can only provide a lower limit for its molecular mass. Accordingly, *engrailed* complex that had been purified by gel filtration was subjected to velocity gradient ultracentrifugation. The gradients were fractionated and the presence of *engrailed* protein in the fractions was detected and quantified using a solid phase radioimmunoassay. The results of this experiment are summarized in Figure 5 and show that the *engrailed* protein is detected throughout the gradient but is concentrated in two peaks at M_r ≃ 10^7 and M_r ≃ 10^7. The removal of Mg^{2+} ions (Figure 5b) from the sample has little effect on the size distribution although a pronounced reduction of the largest material is observed. Exposure to 2 M urea (Figure 5c) causes ~50% of the *engrailed* protein to dissociate from the complex with the M_r ≃ 10^7 peak apparently more sensitive to this denaturation (~70% versus 30%). Treatment with 4 M urea causes about 80% denaturation of the complex.

Purification, DNA binding properties and protein composition of the engrailed complex

A further purification of the *engrailed* complex can be achieved with ion-exchange and affinity chromatography. The *engrailed* complex binds to both DEAE−Sepharose 6B and phosphocellulose at pH 7.6 and is eluted with a salt gradient in a broad peak between 0.3 and 0.5 M KCl. In addition the *engrailed* complex binds quantitatively to DNA
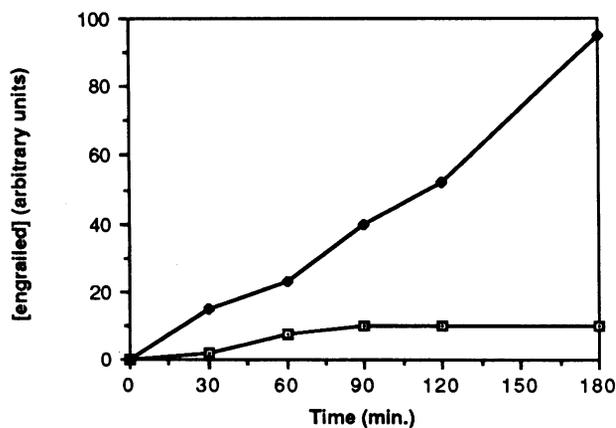
Fig. 4. Accumulation of *engrailed* protein complex and monomer forms in induced HS−EN cells. Nuclear protein extracts were prepared from induced HS−EN cells 30, 60, 90, 120 and 180 min after the cells were returned to 25°C. The extracts were fractionated exactly as described in the legend to Figure 2 and *engrailed* protein in each fraction was quantitated by a solid phase radioimmunoassay. The elution profile of *engrailed* at each timepoint was plotted and the amount of *engrailed* protein present as complex or monomer was determined by integrating the peaks. ♦−♦, low M_r peak; □−□, exclusion volume peak.

Fig. 6. DNA binding of the *engrailed* protein complex. Binding to and elution from DNA cellulose (Alberts and Herrick, 1971). Double-stranded, calf thymus DNA−cellulose column (1 ml) was equilibrated in buffer C + 0.15 M KCl. DEAE ion-exchange purified *engrailed* complex was applied to the column and then eluted with buffer C + 0.3 M KCl (□−□) and 0.6 M KCl (♦−♦). The eluted fractions were assayed for *engrailed* protein.

Table I. Purification *engrailed* protein complex

	protein concentration (mg/ml)	vol (ml)	total protein (mg)	yield purification %	
HS−EN nuclear protein	12.5	8	90	/	/
Sepharose 6B C-L	0.6	10	6.6	80[a]	11
DEAE−Sepharose 6B	0.3	5	1.5	45[b]	(1.9) 21
Phosphocellulose (3.5 ml of DEAE)	0.12	1.2	0.144	35[b]	(3.8) 80

[a]Yield calculated from total protein recovered.
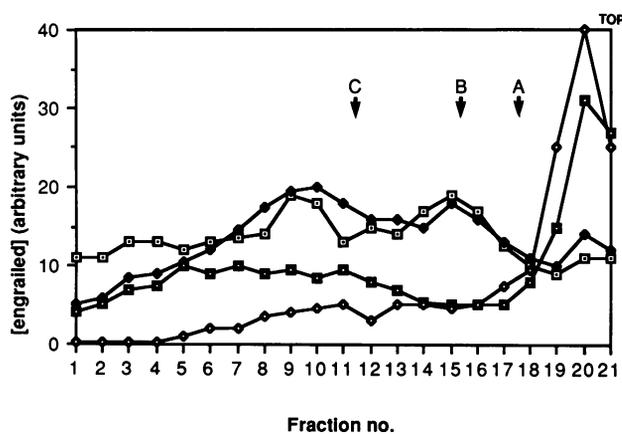[b]Yield calculated by solid phase radioimmunoassays.

Fig. 5. Glycerol gradient ultracentrifugation of the *engrailed* complex. (a) Untreated *engrailed* complex (□−□); (b) Mg^{2+} removed from sample and gradient by the addition of 20 mM EDTA. ♦−♦; (c) *engrailed* complex sample was dialysed into buffer C + 2 M urea for 18 h. Urea (2 M) was also present in the gradient. ■−■; (d) *engrailed* complex sample was dialysed into buffer C + 4 M urea. 4 M urea was also present in the gradient ♦−♦. Molecular mass markers: A, catalase (M_r 2.5 × 10⁶); B, pUC8 plasmid DNA (supercoiled) (M_r 5 × 10⁶); C, bacteriophage λDNA (M_r 3 × 10⁷).

cellulose in 0.15 M KCl and is eluted by 0.3 M KCl (70%) and 0.6 M KCl (30%) (Figure 6). Although this experiment shows that engrailed complex retains the ability to bind DNA it is unclear whether the *engrailed* protein or some other component of the complex mediates this binding. The *engrailed* complex can be purified ∼80-fold by these methods (see Table I for a typical purification), a figure which is limited because the non-uniform nature of the complex results in heterogeneous chromatographic properties. All purification steps do not cause significant dissociation of *engrailed* protein from the complex (results not shown).
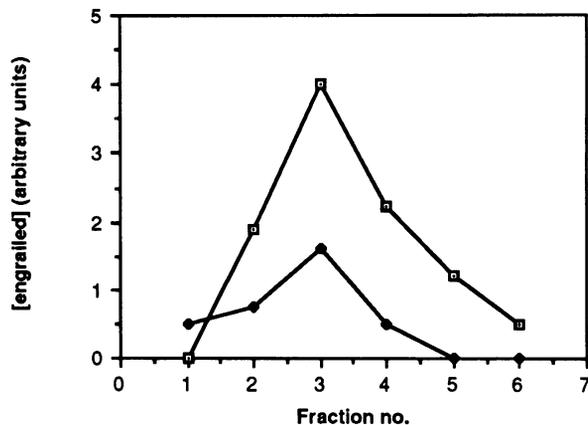
In order to affect a definitive purification of the *engrailed* complex and to determine its average protein composition, purified preparations were radiolabelled with ¹²⁵I and immunoprecipitated with a specific anti-*engrailed* antibody. As a control, a nuclear protein extract of Schneider 2 cells (the parental line of HS−EN which does not express *engrailed*) was purified by gel filtration in Sepharose 6B C-L and radiolabelled in an identical manner to the HS−EN complex. The Schneider 2 extract has a composition that is similar to HS−EN extract (Figure 7a, b) but the anti-*engrailed* antibody does not immunoprecipitate any detectable proteins from it (Figure 7e). By contrast the same characteristic set of proteins are precipitated from all the HS−EN preparations in the same stoichiometry (Figure 7f−h). As a further control, identical precipitation reactions were performed using a polyclonal antibody specific for the homeobox protein *fushi tarazu*. This antibody does not precipitate any protein from either the Schneider 2 or HS−EN preparation (Figure 7i−k).

The *engrailed* complex consists of ∼15 different protein species, two of which (M_r 50 and 60 kd) are predominant being 5-fold more abundant than the others.

Reconstitution of bacterially synthesized engrailed with cell and embryo nuclear protein extracts

As shown above (Figure 7e), specific anti-*engrailed* antibodies do not precipitate any proteins from ¹²⁵I-labelled,
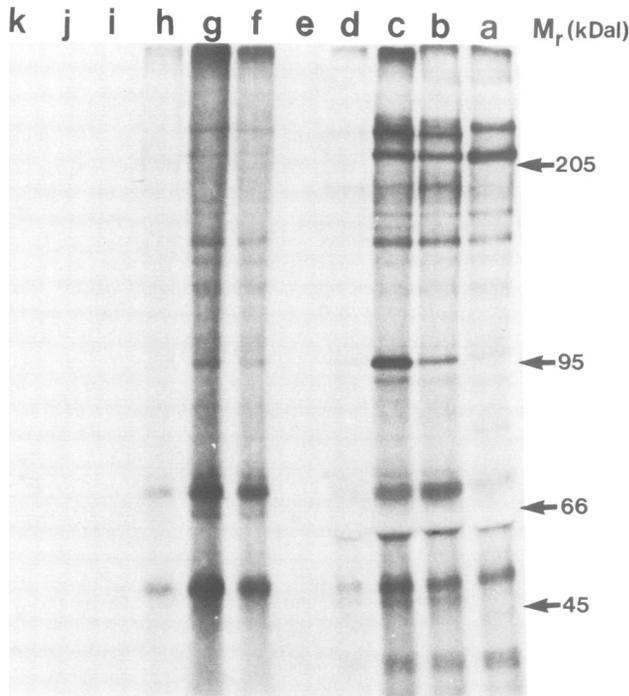
Fig. 7. Immunoprecipitation of purified *engrailed* complex. Samples were fractionated in SDS–PAGE (6%). (a) A nuclear protein extract of Schneider 2 cells was fractionated by gel filtration as described in legend to Figure 2. Protein in the exclusion volume fractions was labelled with ^{125}I. (b) ^{125}I-Labelled *engrailed* complex (see Figure 2). (c) DEAE–Sepharose 6B-purified *engrailed* complex. (d) DNA–cellulose purified *engrailed* complex (see Figure 6). (e–h) Immunoprecipitate of (a–d) with polyclonal anti-*engrailed* antibody. (i–k) Immunoprecipitate of (a–c) with anti-*fushi tarazu* antibody. M, position to which molecular mass standards migrated.

Fig. 8. Reconstitution of *engrailed* protein complex. Samples were separated by SDS–PAGE (7%). (i)(a) ^{125}I-Labelled nuclear protein extract of Schneider 2 cells (see Figure 7a). (b) Immunoprecipitation of (a) with polyclonal anti-*engrailed* antibody. (c) Immunoprecipitation of (a) with anti-*engrailed* antibody. This reaction was preincubated for 30 min with *E.coli engrailed* protein (1 µg). *E.coli engrailed* protein was prepared in a T7 expression system (Studier and Moffat, 1986) (D.Rosen and T.Kornberg; unpublished). (d) Immunoprecipitate of (a) with anti-β-galactosidase polyclonal antibody. The reaction was preincubated with β-galactosidase (3 µg) for 30 min.
(e) Immunoprecipitate of (a) with anti-*fushi tarazu* antibody. (f) *E.coli engrailed* protein (1 µg) added to the reaction but antibody was omitted. (ii) Nuclear protein extract of 2–12-h embryos was purified as described in legend to Figure 3. (a) ^{125}I-Labelled, purified embryo nuclear protein extracts. (b) Immunoprecipitate of (a) with polyclonal anti-*engrailed* antibody. (c) as (b) except that the reaction was pre-incubated with *E.coli engrailed* protein (30 min, 1 µg).
(d) Immunoprecipitate of (a) with anti-*fushi tarazu* antibody.

Schneider 2 cell nuclear protein extract. However, if the extract is incubated with pure, bacterially synthesized *engrailed* protein prior to the immunoprecipitation reaction, a set of proteins identical to those seen in the HS–EN immunoprecipitates are purified [Figure 8(i)a–c]. To show that this reconstitution of the *engrailed* complex is not an artefact of immune complex formation, β-galactosidase was added to a control reaction and then precipitated with an affinity purified rabbit anti-β galactosidase antibody. The ^{125}I-labelled Schneider 2 protein extract in the reaction did not become associated with these β-galactosidase immune precipitates (Figure 8(i)d). The ability to reconstitute the *engrailed* complex in this way shows that the proteins with which *engrailed* associates are present normally in the cell and that their formation is not catalysed by *engrailed* protein expression. In order to determine whether bacterial *engrailed* protein can also reconstitute with soluble protein components in the embryo, a nuclear protein extract of 12-h embryos was chromatographed in a column of Sepharose 6B-C-L and the protein that eluted in the exclusion volume of the column was labelled with ^{125}I. This labelled embryo nuclear protein extract was used in an immunoprecipitation reaction with the anti-*engrailed* antibody. In the absence of exogenous *engrailed*, little protein can be detected in the immuno-precipitate [Figure 8(ii)a,b]. However, when the embryo protein preparation is preincubated with bacterial *engrailed* protein a subset of proteins are purified by the immuno-precipitation reaction [Figure 8(ii)c]. Of the 11 species detected in the reconstituted embryo extract, five appear to
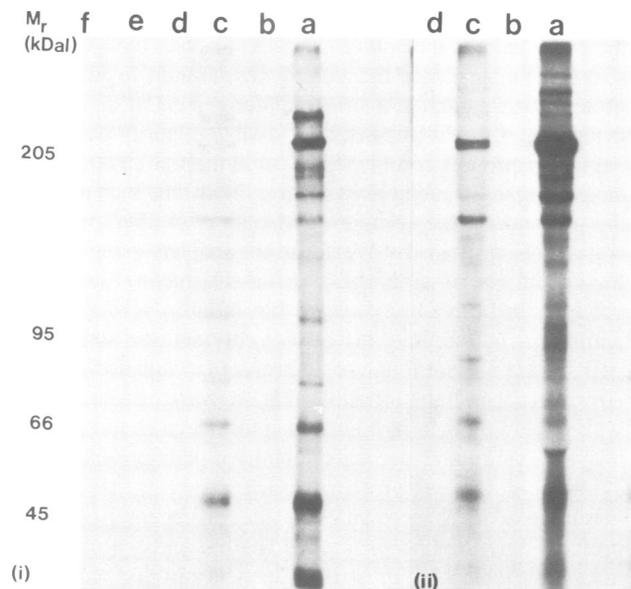
have counterparts in the HS–EN cell derived *engrailed* complex, including the M_r 50- and 60-kd proteins. Thus the developing embryo also has soluble protein complexes that are able to interact stably with the *engrailed* protein and the average composition of these complexes is related to but distinct from the *engrailed* nuclear protein complex of HS–EN cells.

Discussion

In this paper we have shown that the *engrailed* protein functionally expressed in a *Drosophila* cell line and in developing embryos is able to make specific and stable interactions with other soluble nuclear proteins. These *engrailed* protein complexes do not contain any polynucleotide components and are stable in conditions that dissociate non-specific ionic and hydrophobic protein–protein interactions. Furthermore purified complexes are found to be constituted of a small subset of total soluble nuclear proteins and can bind quantitatively and reversibly to DNA results that further confirm the specificity of the observed interactions. Analytical velocity gradient ultracentrifugation of the complexes shows them to be both large and hetero-geneous in the size range M_r ~ 10^7–10^8 D and specific immunoprecipitation reveals that the constituent proteins

taken together in a unitary stoichiometry cannot account for their measured molecular masses. These two findings strongly suggest that the complexes contain a polymeric protein species such as a structural component of the nuclear cytoskeleton. In this regard, it is possible to purify unlabelled *engrailed* protein complexes by immunoprecipitation with an anti-*engrailed* rabbit polyclonal antibody and then to detect the presence in the complexes of both *engrailed* and other component proteins by Western blot analysis using heterologous antibodies as probes. Using this method *engrailed* protein can be readily detected but tubulin, actin, nuclear lamin b, α subunit of DNA polymerase and RNA polymerase are not found to be present (results not shown). However, it remains possible that *engrailed* protein binds with a soluble fraction of nuclear cytoskeletal elements other than nuclear lamin b actin and tubulin or perhaps with a non-cytoskeletal nuclear protein polymer.

Recent work has found that the divergent homeobox proteins *engrailed*, *even skipped*, *paired* and *zen* produced in bacterial cells bind *in vitro* to identical sites located within the promoter regions of the *engrailed* and *even skipped* genes, possibly with varying affinities (Hoey and Levine, 1988). These results have been interpreted to mean that the different homeobox proteins act upon the same set of regulatory sites and that characteristic patterns of gene expression are set up either because the proteins have differing affinities for these sites or because different combinations of homeobox proteins recognize qualitatively different DNA sequences.

The results reported here suggest a third possibility: that the specificities, affinities or functions of *engrailed* and other homeobox proteins are modified and differentiated by their interactions with other nuclear protein complexes.

Materials and methods

Cell culture techniques

Schneider 2 and HS−EN cell lines were grown in suspension culture at 25°C in Schneider *Drosophila* medium supplemented with 10% fetal calf serum. For immunofluorescence microscopy samples of cultures (4 × 10⁶ cells/ml) were applied to glass coverslips and allowed to settle for 10 min. The cells were fixed *in situ* with a solution of 3.7% formaldehyde, 0.1% NP40, 25 mM Tris pH 8, 150 mM NaCl for 10 min at 25°C and then washed in 25 mM Tris pH 8, 150 mM NaCl, 0.1% NP40, 1% bovine serum albumin (BSA) (3 × 5 min) (buffer A). The fixed, immobilized cells were incubated with an appropriate dilution of antibody in buffer A for 1 h and then washed in buffer A (3 × 5 min). The cells were then incubated with rhodamine conjugated secondary antibody (Capel, 1.100 dilution in buffer A). The DNA specific dye DAPI (4′,6′-diamidino-2-phenyl indole) was added to a final concentration of 5 μg/ml for 2 min and then the cells were washed (5 × 10 min) in buffer A. Finally the coverslip was mounted and viewed.

Antibodies

Monoclonal anti-*engrailed* antibodies were prepared as described (Karr *et al.*, in preparation). Polyclonal anti-*engrailed* antibody were prepared by inoculating rabbits with a β-galactosidase−*engrailed* fusion protein. Antiserum from the rabbits was precipitated by the addition of ammonium sulphate to 60%, redissolved in phosphate buffered saline [3 mM NaH₂PO₄, 7 mM Na₂HPO₄, 150 mM and NaCl (PBS)] and dialysed against the same buffer. Specific anti-*engrailed* antibodies were selected on a column of Affi-gel 10 (Biorad) to which purified *Escherichia coli* produced *engrailed* protein had been coupled and then were eluted from the column with 0.1 M glycine pH 3. The eluate was immediately neutralized by the addition of 1/20 volume of 1 M Tris pH 8 and then dialysed against PBS. Anti-*fushi tarazu* antibody was a gift of Dr S.Carroll (Carroll and Scott, 1985).

Preparation of nuclear protein extract from embryos and culture cells

All procedures were performed at 4°C. Embryos were collected on corn meal agar plates, washed in 0.7% NaCl, 0.04% Triton X-100 and dechorionated by exposure to half strength bleach for 90 s. Embryos were suspended in 15 mM Hepes pH 7.6, 10 mM KCl, 5 mM MgCl₂ 0.1 mM EDTA (ethylene diamine tetra-acetic acid), 0.5 mM EGTA (ethylene β amino ethyl ether) *N,N,N′,N′*-tetra-acetic acid, 1 mM phenyl methyl sulphonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 2 μg/ml leupeptin (4 ml/g embryos; buffer B), and homogenized with four strokes of a motorised Teflon pestle and eight strokes of a Dounce 'B' type pestle. The homogenate was strained through two layers of mira cloth (Calbiochem) and the white suspension was centrifuged at 2000 g for 10 min. The soft white nuclear pellet was resuspended in buffer B overlaid on cushions of buffer B + 0.8 M sucrose and centrifuged at 1000 g for 10 min.

Cell cultures were grown to a density of 5 × 10⁶ cells/ml and harvested by centrifugation (1000 g for 10 min). The cells were resuspended in 1/80 volume of ice-cold buffer B and centrifuged at 1000 g for 10 min. The cell pellet was resuspended in buffer B, lysed with 25 strokes of a Dounce type 'B' pestle and nuclei were collected by centrifugation (1000 g for 10 min). Nuclear pellets of both embryos and cell cultures were resuspended in buffer B, adjusted to 150 mM KCl and lysed by the addition of ammonium sulphate to a concentration of 0.4 M. The nuclear lysates were centrifuged at 120 000 g for 60 min and the soluble protein in the supernatant was precipitated by the addition of 60% ammonium sulphate. The ammonium sulphate precipitate was redissolved in 25 mM Hepes pH 7.6, 100 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF 10% glycerol (Buffer C) at a concentration of 10−30 mg protein/ml.

Protein determination

A dye binding assay was used (Bradford, 1976).

Glycerol gradient ultracentrifugation

Samples (0.25 ml) were applied to preformed 15−40% glycerol gradients (14 ml) in buffer C + 0.6 M KCl and centrifuged in a Beckman SW40 rotor at 160 000 g for 14 h at 4°C. The gradients were fractionated by pumping from the bottom of the gradient. Size standards were run in duplicate gradients.

Western blot and solid phase radioimmunoassay

Western blot analysis was performed as previously described (Towbin *et al.*, 1979). For rapid quantitation, samples of column fractions were applied to a nitrocellulose filter (Whatman) either directly or using a 'dot blot' apparatus (Schleicher and Schuell). The filter paper was then blocked in a solution of 1% bovine serum albumin (Sigma) 25 mM Tris pH 7.6, 150 mM NaCl, 0.1% NP40, 5 mM EDTA (10 min at 25°C; buffer D). The filter was incubated with monoclonal anti-*engrailed* antibodies (1/100 dilution in buffer D) at 25°C for 1−4 h then washed (3 × 5 volumes buffer D) and incubated with ¹²⁵I-labelled second antibody (2 μCi/ml) (Amersham) for 1 h. Finally, the filter was washed in buffer D supplemented with 0.1% SDS and 0.5% NP40 (5 × 10 min). The dots were then cut out and quantitated using a Beckman gamma counter. The assay was calibrated using double dilutions of an *engrailed* protein standard.

Radiodination and immunoprecipitation of nuclear protein samples

Protein samples for radiodination were dialysed exhaustively into 50 mM Tris−HCl pH 7.6, 100 mM KCl, 5 mM MgCl₂, 10% glycerol (chromatographic grade). To 100 μl of the protein sample, 10 μl of Na¹²⁵I (10 mCi/ml, Amersham) and 10 μl of fresh chloramine T solution (5 mg/ml) were added. The reaction was allowed to proceed for 90 s at room temperature and then 50 μl at saturated tyrosine solution was added. After a further 90 s the reaction mixture was loaded on a 2.0-ml column of Sepharose 6B C-L (Pharmacia) which had been equilibrated in buffer C. The column breakthrough fraction was collected and immediately frozen in liquid nitrogen. For immunoprecipitation reactions, 25 μl of ¹²⁵I-labelled protein was added to 400 μl of buffer C supplemented with 10 mg/ml BSA and 0.1% Triton X-100. Antibody solution (20 μl) was added and the solution was incubated at 4°C for 30 min. Then, 50 μl of a 50% slurry (in buffer C) of protein A−Sepharose 4B (Pharmacia) was added and incubated for 30 min at 4°C. Finally the Sepharose beads were washed in buffer C supplemented with 0.15% sodium deoxycholate, 0.1% Triton X-100, 0.05% sodium dodecyl sulphate (SDS) (5 × 10 min) and buffer C alone (3 × 1 min). SDS−PAGE sample buffer (50 μl) (125 mM Tris pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 4 M urea and 0.0025%

bromophenol blue) was added to the beads and the immunoprecipitated protein was removed by boiling the samples for 5 min. The samples were then analysed by SDS−PAGE (Laemmli, 1970).

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