TWO-DIMENSIONAL GEL ANALYSIS AFTER REMOVAL OF MAJOR PROTEINS REVEALS STAGE-DEPENDENT PROTEINS IN EARLY INSECT DEVELOPMENT

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1. Introduction

Protein synthesis occurring during development, or in the various tissues of eucaryotic organisms, is commonly analysed by high resolution two-dimensional (2D)-gel electrophoresis [1] or by immunological techniques [2]. Both techniques, however, are limited in detecting small amounts of proteins in the presence of abundant other proteins [2,3]. Consequently, few tissue-specific and/or stage-dependent proteins have been reported [3–8]. For insect embryogenesis, neither qualitative nor even major quantitative differences have been observed in the patterns of proteins synthesized before the onset of embryonic mRNA synthesis around blastoderm formation [2,9,20].

This paper describes removal of major proteins by antibody precipitation followed by 2D-gel analysis of the non-precipitated proteins. Using this method, we observed differential protein synthesis at two early stages of insect development, not detected by either immunological techniques or 2D-gel analysis [2,9,10]. Moreover, our results suggest translational control for maternal mRNA already present in the insect egg.

2. Materials and methods

2.1. *In vivo radioactive labeling of proteins in* Smittia *embryos*

Eggs and embryos were obtained from our laboratory strain of the chironomid midge *Smittia* sp. [10]. Embryonic stages were defined by time after deposition (at 21°C) and by morphological criteria [11]:
Stage P_2 (intravitelline cleavage, 2 pole cells; 3–4 h after deposition);
Stage P_4 (intravitelline cleavage, 4 pole cells; 4–5 h after deposition);
Blastoderm stages (formation of cell layer; 8–14.5 h after deposition).

To label newly synthesized proteins, embryos were permeabilized at stages P_2 or P_4, and subsequently incubated (for 25 min) in basic salt medium containing 150 μCi [^{35}S]methionine (870 Ci/mmol; NEN) as detailed in [10].

2.2. *Preparation of IgG-antibodies against major* Smittia *proteins*

Protein from *Smittia* eggs and early embryos (≤2.5 h after deposition) were extracted [10]. Extracts (1.8 mg total protein) were injected subcutaneously into rabbits [12] to raise antibodies. Antisera were collected [12], dialysed against 0.028 M NaCl, 0.02 M Tris–HCl (pH 8.0), and fractionated on a DEAE Affi-Gel dextran blue column (Bio-Rad Labs., Philadelphia) as suggested by the manufacturer. The IgG-fraction was dialysed against diluted (1:3 with distilled water) phosphate-buffered saline [13] and concentrated 3-fold (antibody solution; 8 mg/ml).

2.3. *Immunoprecipitation and electrophoresis*

Labeled or unlabeled *Smittia* embryos, *Drosophila* embryos, or *Smittia* or *Chironomus thummi* females were sonicated in 50 μl 0.001 M Tris–HCl (pH 7.5) and processed as in [10]. Protein extracts were adjusted to 0.85% NaCl and immunoprecipitated with antibody solution (see section 2.2) [12]. Alternatively, the *Staphylococcus* protein-A method in [13] was used. Immunoprecipitates (precipitate) were

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analysed on 9.2% SDS slab-gels [14] or 2D-gels (see below). The supernatants (non-precipitated proteins) were desalted over a 3 ml Sephadex G-50 column (Pharmacia), eluted in 1 ml distilled water, lyophilized and dissolved in 50 μl lysis buffer [15]. 2D-Gel electrophoresis [15] was modified using a 9.2% SDS slab-gel for the second dimension and fluorography as in [10]. The double diffusion Ouchterlony test was as in [12].

3. Results and discussion

3.1. *Double diffusion test and immunoprecipitation of protein extracts from stage P_2 embryos*

IgG-antibodies made against protein extract from early embryos and eggs of *Smittia* sp. were tested in Ouchterlony plates. They reacted with protein extract from *Smittia* embryos and females, and with protein extracts from two different insect species (fig.1a). The fusion of the major precipitin arcs indicated crossreactivity with all protein extracts tested (fig.1a).

Immunoprecipitation was optimized in a dilution series. Constant amounts of protein extract (5 μg; 250 stage P_2 embryos) were reacted with various amounts of antibody solution (1 nl–100 μl). Protein extract, precipitates and non-precipitated proteins were analysed by SDS gel electrophoresis (parts shown in fig.1b–f). Under optimal conditions (5 μl antibody solution), only a few protein bands were obtained with non-precipitated proteins (fig.1e) while the banding pattern from the precipitate (fig.1e) corresponded to that from the protein extract (fig.1b). However, quantitative precipitation of all proteins in the extract could not be achieved, since excess of antibodies and/or antigens in the reaction mixture (non-uniform antibody solution; variation in quantity of different antigens) prevented simultaneous equivalence point precipitation. The use of IgG–antibody solution together with *Staphylococcus* protein A–antibody adsorbent obviated the need of equivalence point titration for quantitative precipitation [13].

Protein extracts containing newly synthesized labeled proteins (5 μg; trichloroacetic acid-precipitable radioactivity 200 000 cpm) were reacted with antibody solution (5 μl; 30 min; 37°C; reaction vol. 50 μl) and adsorbed to the protein-A matrix as in [13]. Reproducible precipitation (94–97% label in the precipitate; 12 expt) was obtained with the same frozen aliquot of antibody solution. A broader range of precipitated label (83–97%) was observed with different aliquots. Furthermore, after various cycles of freezing and thawing of the original aliquot or with antibody solution from earlier bleedings, much less labeled protein (40–70% of the radioactivity) was precipitated from the same protein extract. For comparative studies on stage-dependent protein synthesis (see below), the same aliquot of antibody solution was used to remove major proteins from the different protein extracts.

3.2. *Stage-dependent protein synthesis during early insect development*

In *Smittia*, 2D-gel analysis revealed only 8 out of

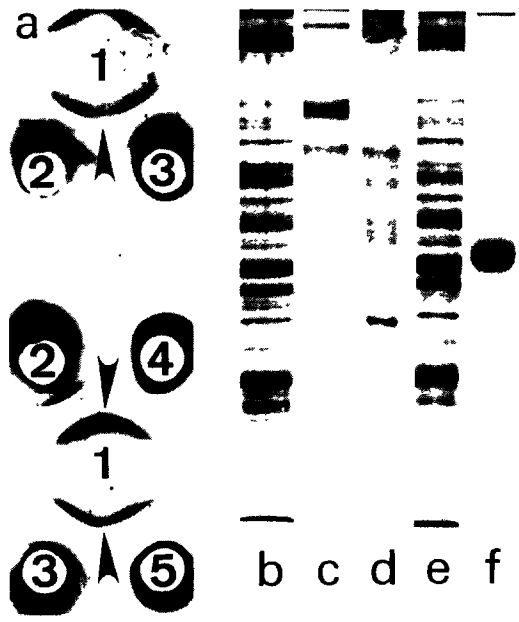

Fig.1. Double diffusion test and immunoprecipitation of *Smittia* embryo proteins at stage P_2. Protein extracts, 10 μg; Antibody solution, 2 μl. (a) Ouchterlony plates: (1) antibody solution; (2–5) protein extracts from (2) *Smittia* embryos (stage P_2); (3) *Smittia* females, (4) *Drosophilia* embryos, (5) *Chironomus* thummi females. Diffusion period, 48 h [12]. Arrowhead indicates fusion of the major precipitin arc. (b–f) SDS gel patterns: (b) protein extract from *Smittia* embryos (5 μg protein; 250 embryos); (c–e) Precipitate from 10 μg protein extract (500 embryos) with various amounts of antibody solution: (c) 0.5 μl antibody solution, total precipitate; (d) 50 μl antibody solution, half of the precipitate; (e) 10 μl antibody solution, half of the precipitate; (f) non-precipitated proteins (total supernatant proteins) of immunoprecipitate. Note: few proteins are left in the supernatant.

~450 proteins with altered synthesis during development between stage P_2 and stage M_2 [10]. Similar results were obtained with *Drosophila* embryos [2,9].

In accordance with the results in [10], virtually no differences could be observed between fluorographs of precipitated proteins synthesized at stage P_2 and P_4, respectively (not shown). Furthermore, these fluorographs were overexposed after 3 weeks exposure (fig.2a; [10]). In contrast, 6 weeks exposure was needed to detect protein spots on fluorographs of the non-precipitated proteins. Fewer than 30 faint protein spots were observed at stage P_2 (fig.2a). Most of these proteins were also detected in the precipitate (circles; fig.2b) which indicated incomplete but efficient removal of the newly synthesized proteins. At stage P_4, however, 23 additional protein spots appeared in the non-precipitated proteins (fig.2c). These proteins were also observed with a different batch of embryos and a different aliquot of the antibody solution (fig.2d). However, none of these proteins could be detected at stage P_2 under identical conditions.

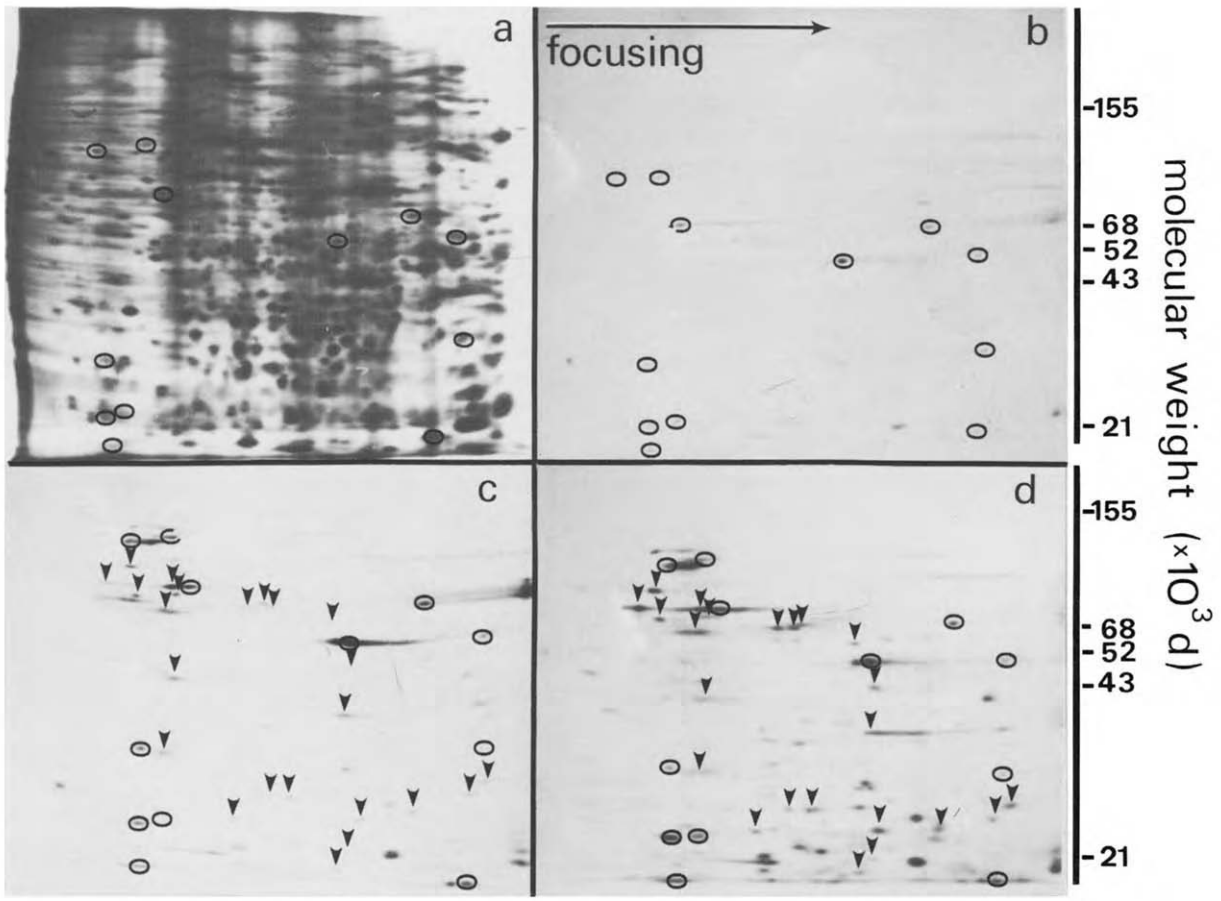

Fig.2. 2D-gel analysis of immunoprecipitated and non-precipitated *Smittia* proteins. Protein extracts (5 μg) from 250 [^{35}S]methionine-labeled *Smittia* embryos (200 000 cpm) at stage P_2 and stage P_4 were precipitated under standardized conditions (see text). (a) Precipitate and (b–d) non-precipitated proteins were separated by 2D-gel electrophoresis followed by fluorography [10]. Circles represent non-precipitated proteins also present in the precipitate and can be used as landmark spots. (a) Overexposed fluorograph of the immunoprecipitate (192 000 cpm) from stage P_2 embryos. Exposure period, 3 weeks. (b) Fluorograph of non-precipitated proteins (7500 cpm) from stage P_2 embryos. Note that a 6 week exposure period revealed faint protein spots only. (c) Fluorograph of non-precipitated proteins (11 600 cpm) from stage P_4 embryos precipitated with the same antibody aliquot as in (b). Arrowheads indicate protein spots not observed at stage P_2. Exposure period, 6 weeks. (d) Fluorograph of non-precipitated proteins (14 300 cpm) from stage P_4 embryos. Note: Embryo batch and antibody aliquot different from (c); Reproducibility of the 'new' spots (arrowheads). Exposure period, 8 weeks.

The appearance of these new proteins at stage P_4 indicated stage-dependent protein synthesis at an early point in insect development. Their stage dependency was particularly interesting in relation to the role of maternal mRNA for protein synthesis in the absence of embryonic transcription before blastoderm formation ([10], data on *Smittia).* Our experiments suggest differential utilization of 'stored' maternal mRNA [16] at early embryonic stages [17, 18] and, in turn, translational control for protein synthesis during early development of an insect.

4. Conclusions

Removal of major proteins by immunoprecipitation followed by 2D-gel analysis of the non-precipitated proteins revealed qualitative differences in the pattern of newly synthesized proteins not detected by either of the two techniques alone. Immunoprecipitation was the critical step of our analysis and was dependent upon the antibody preparation used. Some protein precipitation might be due to high crossreactivity of antibodies with various proteins in the extracts, but reproducible results were obtained under standardized conditions. The basic method described here might be adapted to other organisms for detection of minor translation products as described here.

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