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Studies on embryonic diapause in the *pnd* mutant of the silkworm, *Bombyx mori*

V. Identification of a pnd^+ gene-specific protein

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Summary. Two-dimensional gel electrophoresis has been used to analyse patterns of proteins synthesized in the eggs from the *Bombyx* mutant *pnd*, whose homozygous embryo never enters diapause owing to a genetic defect. At the middle to late stage of gastrulation the diapause type of the heterozygous embryo, derived from a homozygous *pnd* female mated to a wild-type male, synthesizes eight proteins which are not detected in the homozygous *pnd* embryo. To examine the relationship between embryonic diapause and the appearance of the heterozygote-specific proteins, the pattern of proteins synthesized in the heterozygotes of the diapause type was compared with that in heterozygotes, which were artificially altered so that they would continue development. Only one of the eight heterozygote-specific proteins was constitutively synthesized according to the embryonic genome, irrespective of their developmental state, whereas appearance of the remaining seven proteins was exclusively dependent on their developmental nature. This finding strongly suggests that the unique protein might result from the expression of the pnd^+ gene, and the other proteins might be synthesized along with diapause initiation in the heterozygotes. The possible role of the putative pnd^+ gene-specific protein at the onset of embryonic diapause is discussed in relation to the action of the diapause factor, which predetermines embryonic diapause by affecting the developing oocytes.

Key words: Protein synthesis – Gene-product analysis – Two-dimensional gel electrophoresis – *Bombyx* silkworm

Introduction

The silkworm, *Bombyx mori*, overwinters at the late gastrula stage of embryogenesis (Toyama 1902; Sonobe et al. 1986). In the wild-type silkworm (bivoltine race), embryonic diapause is predetermined by the proteinaceous hormone (Sonobe and Ohnishi 1971; Sonobe 1974), called the diapause factor, secreted from the suboesophageal ganglion of the pharate adult, which acts on the oocytes developing in the ovarioles of the pharate adult (Fukuda 1951, 1963). The pigmented and non-diapausing egg mutant (*pnd*) of the silkworm, a single recessive mutation of a gene on the 11th chromosome (Yamamoto et al. 1978), has a defect as a result of which the embryo never enters diapause, even though the eggs have been exposed to the diapause factor during oogenesis. However, the heterozygous embryo from the homozygous *pnd* female mated to the wild-type male can enter diapause (Katsumata 1968), which suggests that the embryonic defect caused by the *pnd* mutation is repairable after fertilization by the action of the paternal pnd^+ gene. Thus it has been proposed that two mechanisms are involved in the process determining embryonic diapause in the silkworm: one is a process predetermined by the diapause factor during oogenesis, and the other is a process determined by a genetic factor during embryogenesis. The genetic defect in the *pnd* mutant might be involved in the process of diapause determination, which operates after fertilization, but not in the responsiveness of the ovaries to the diapause factor (Yoshitake and Hashiguchi 1969; Sonobe 1984).

Recently, we found that diapause in the heterozygous embryo of the *pnd* mutant is associated with a large increase in the quantity of the products of anaerobic metabolism, such as polyols, lactate and alanine, as compared with the homozygous *pnd* embryo, which develops as the non-diapause type (Sonobe 1984; Sonobe and Okada 1984). Furthermore, it was also demonstrated that the rate of DNA synthesis increases gradually with development after early gastrula in the homozygote, but in the heterozygote it begins to decrease significantly at the middle stage of gastrulation, and the embryo finally enters diapause at late gastrula (Sonobe et al. 1986). The biochemical dividing point between the homozygote and heterozygote is detected 36 h after oviposition, which suggests that the paternal pnd^+ gene has already participated in a process controlling these biochemical changes by this time. At present there is very little knowledge of the biochemical function and characterization of the pnd^+ gene-specific product, even though it is obvious that the role of the pnd^+ gene is developmentally interesting, as described above. The present study was designed to define the effect of the pnd^+ gene on the rate of protein synthesis and to identify the pnd^+ gene-specific product on two-dimensional polyacrylamide gels by comparing the pattern of proteins synthesized during development of the homozygote and heterozygote of the *pnd* mutant.

Materials and methods

Experimental animals. Homozygous *pnd* mutants were incubated at 25 °C under continuous illumination during their embryonic development. Under these conditions, when ho-

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mozygous *pnd* females were mated to homozygous *pnd* males, all of the resulting eggs (homozygotes) became the non-diapause type, and the larvae hatched 11 days after oviposition. On the other hand, when the homozygous *pnd* females were mated to wild-type males (Chinese 124, which has a common genetic background with the *pnd* mutant, except for the mutant gene *pnd*), all of the resulting eggs (heterozygotes) became the diapause type.

The suboesophageal ganglion of the homozygous *pnd* pupa was removed soon after larval-pupal ecdysis. The homozygous *pnd* female, which has been deprived of the suboesophageal ganglion, laid non-diapause eggs exclusively, even though she was mated to a wild-type male.

In order to change prospective diapause eggs to the non-diapause type, 24-h-old heterozygotes were soaked in HCl solution (specific gravity, 1.11 at 15 °C) at 24 °C for 70 min, and then thoroughly washed in running water and dried. The larvae hatched from HCl-treated eggs 11–12 days after oviposition.

To sample closely synchronized eggs, the period of oviposition was limited to less than 1 h. Pooled eggs were kept at 25 °C until the start of the experimental treatment.

Radioactive labelling and determination of radioactivity. At the appropriate developmental stages in early embryogenesis, each egg was injected with 20 nl aqueous solution containing 0.3 μCi [^{35}S]methionine (specific activity, 1,340 Ci/mmol; Amersham). The method of injection was described in detail in our previous paper (Sonobe et al. 1986). The injected eggs were incubated in a humid chamber for 3 h at 25 °C. The incubation was stopped by freezing the eggs at −70 °C. Ten eggs were homogenized with 50 μl lysis solution containing 9.5 M urea, 2% (w/v) Nonidet P-40, 5% (v/v) 2-mercaptoethanol and 2% (v/v) ampholines (pH range 3.5–10, LKB). The homogenate was centrifuged at 800 rpm for 5 min (Hitachi, 05PR) to remove the chorion. The supernatant was incubated at 30 °C for 60 min and the solubilized sample was then centrifuged at 3,000 rpm for 15 min. We used 3 μl of the resulting supernatant to determine the amount of [^{35}S]methionine incorporated into trichloroacetic acid (TCA)-insoluble material. The remainder of the supernatant was frozen at −70 °C until electrophoresis. To count the radioactivity of TCA-insoluble material, 3-μl aliquots were placed on filter discs (Whatman 3MM); the discs were soaked in 10% TCA at 0 °C for 10 min and were then washed in 10% TCA at 100 °C for 5 min, and once each in ethanol and ether. The discs were dried and counted in a toluene-based scintillation mixture in an Aloka scintillation counter (LSC-703).

Two-dimensional electrophoresis of proteins. The newly synthesized proteins were analysed by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE; O'Farrell et al. 1977). The first dimension, non-equilibrium pH gradient electrophoresis, was run at 500 V for 4 h (2,000 Vh) at 25 °C. The second dimension, sodium dodecylsulphate (SDS)-PAGE (12%), was run with 23 mA constant current. After electrophoresis in the second dimension, the gels were fixed with 1 M salicylate solution and dried. Radioactive proteins were located by salicylate fluorography (Chamberlain 1979) at −70 °C with Kodak film (XAR-5). The cpm applied in each first dimension column are specified in the figure legends. The following were used for molecular weight markers: ovotransferrin (78,000), bovine serum albumin (66,000), ovalbumin (45,000), chymotrypsinogen A (26,000) and myoglobin (17,000). To measure the pH gradient in the non-equilibrium electrophoresis, the first dimension gel was cut into 1-cm sections, which were eluted by incubation with 1 ml deionized distilled water in capped vials for 3–12 h. The pH was then measured at room temperature. The pH was stable during the incubation period.

Results

Protein synthesis during early embryogenesis

In our previous experiments (Sonobe and Okada 1984), it was demonstrated that most of the free amino acids in the homozygote and heterozygote of the *pnd* mutant vary in amount during embryogenesis, but three (glutamic acid, methionine and histidine) are almost constant throughout early embryogenesis. In addition, these three amino acids did not show a noticeable quantitative difference between homozygote and heterozygote during early development. Glutamic acid and histidine were always comparatively plentiful, suggesting that labelling using these amino acids would be less efficient in vivo. However, the pool size of methionine was small throughout development. Consequently, radioactive methionine was used to study the changing pattern of protein synthesis during early development.

In Fig. 1 the incorporation of [^{35}S]methionine into protein at different stages in early embryogenesis is shown. There is no appreciable difference between the homozygote and heterozygote throughout the stages of syncytial blastoderm (3-h embryo), cellular blastoderm (12-h embryo) and early gastrula (24-h embryo). A significant difference in the rate of protein synthesis between the homozygote and heterozygote was detected from the middle stage of gastrulation (36-h embryo). In the homozygote, the rate of protein synthesis increased gradually from late gastrulation (48-h embryo) to early organogenesis (72-h embryo) and then levelled off during organogenesis (96-h embryo). On the

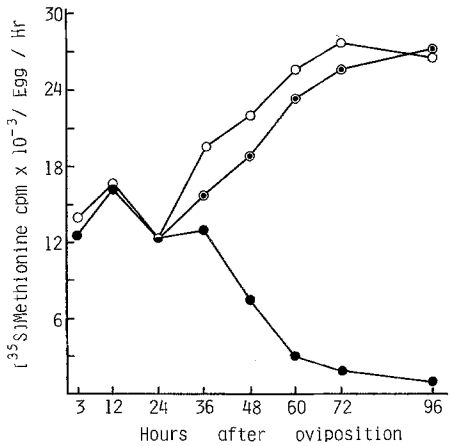


Fig. 1. Incorporation of [^{35}S]methionine into proteins at different stages of early development in the homozygote and heterozygote of the *pnd* mutant. o–o homozygotes; ●–● heterozygotes; ⊙–⊙ HCl-treated heterozygotes (see Materials and methods). Eggs were injected with 20 nl [^{35}S]methionine and incubated for 3 h at 25 °C. The TCA-insoluble material was collected on the filter discs, and the radioactivity was counted (see Materials and methods). Similar incorporation patterns were observed in three separate experiments

other hand, in the heterozygote, the rate of protein synthesis decreased gradually during the middle-late gastrula stage (48-h embryo) and fell to a very low level in the late stage of gastrulation (72-h embryo), suggesting that the embryo had entered diapause at that stage (for further details of morphological changes, see Sonobe et al. 1986). When heterozygotes were activated by HCl-treatment to prevent diapause initiation, the rate of protein synthesis increased remarkably. The increasing rate of protein synthesis was almost the same as that of the homozygotes except for a slight lag (Fig. 1).

Two-dimensional gel patterns of proteins synthesized in early embryogenesis

From the changing patterns in the rate of protein synthesis between homozygote and heterozygote (Fig. 1), it is clear that the paternal *pnd*$^+$ gene has been activated in the heterozygote during early embryogenesis, and the product exerts its effect at least 36 h after oviposition. Therefore, it was expected that the *pnd*$^+$ gene-specific product would be identifiable at 36 h after oviposition by a difference in the two-dimensional gel patterns between the homozygote and heterozygote. Example of the patterns of [^{35}S]methionine-labelled proteins extracted from the homozygote and heterozygote during early development are shown in Fig. 2. Some of the [^{35}S]methionine-labelled proteins varied with development in both the homozygote and heterozygote, suggesting that some genes were expressed differentially during development. However, since the purpose of this experiment is mainly to analyse the difference in the pattern of protein synthesis associated with differences in the embryonic genome, proteins which change in common in the homozygote and heterozygote during embryogenesis are less important than proteins which differ between the two types of embryos at corresponding periods of development. To assist in comparing pattern of proteins synthesized, the proteins which are specific or predominant to either the homozygote or the heterozygote at the corresponding period of development were numbered.

At 12 and 24 h after oviposition, no remarkable differences in the patterns of [^{35}S]methionine-labelled proteins were detected between the homozygote and heterozygote (compare a with b, and c with d in Fig. 2). However, at 36 h after oviposition, eight differences in newly synthesized proteins were detected between the homozygote and heterozygote: three proteins (spots 4, 5, 8) disappeared in the heterozygote, although the homozygote continued to synthesize these three proteins at that period, and five new proteins (spots 3, 12–15) appeared exclusively or predominantly in the heterozygote. At 48 h after oviposition, three proteins (spots 1, 2, 11) newly appeared in the heterozygote in addition to the five new proteins which were synthesized at 36 h. The eight proteins (spots 1–3, 11–15) which appeared exclusively or predominantly in the 36- or 48-h heterozygote will henceforth be called generically heterozygote-specific proteins. In the 48-h heterozygote, four proteins (spots 6, 7, 9, 10) which were detected at 36 h disappeared, although they continued being synthesized in the 36- and 48-h homozygotes. In the homozygote, some proteins (spots 4, 5, 8 at 36 h, and 4–10 at 48 h) appeared which were not detected in the heterozygote at the corresponding periods of development. However, we did not regard them as proteins specific for the homozygote, since they appeared at an earlier stage of the heterozygote, and then disappeared at 36 or 48 h after oviposition. Therefore, the failure of these proteins to disappear may be a useful indicator in embryonic development.

To examine whether the putative *pnd*$^+$ gene-specific protein was among the eight heterozygote-specific proteins, the developmental fate of the heterozygotes was changed artificially to the non-diapause type by HCl-treatment (see Materials and methods), and the pattern of proteins synthesized in HCl-treated eggs was compared with that of the untreated heterozygote. It has been suggested that the HCl-treatment enhances oxygen permeability of the chorion, so that prospective diapause eggs cease entering diapause (Sonobe et al. 1979). Therefore, it was expected that the *pnd*$^+$ gene-specific protein would be produced according to the genotype, even though the developmental fate of the embryo was altered. If so, a *pnd*$^+$ gene-specific protein might be distinguishable among the eight heterozygote-specific proteins. Examples of the patterns of [^{35}S]methionine-labelled proteins extracted from HCl-treated heterozygotes are shown in Fig. 3. Four (spots 3, 12–14) out of five heterozygote-specific proteins were hardly detectable in the 36-h HCl-treated heterozygote (12 h after HCl-treatment), but spot 15 was quite prominent in both the treated and untreated heterzygotes (compare Fig. 3a with Fig. 2f). Furthermore, three new proteins (spots 4, 5, 8) appeared in the 36-h HCl-treated heterozygote. The appearance of these new proteins was comparable with the pattern of proteins synthesized in the 36-h homozygote (compare Fig. 3a with Fig. 2e). In the 48-h HCl-treated heterozygote, seven (spots 1–3, 11–14) out of the eight heterozygote-specific proteins which had been detected in the untreated 48-h heterozygote were no longer found. Only spot 15 was still synthesized in both the 48-h treated and untreated heterozygotes (compare Fig. 3b with Fig. 2h). Furthermore, at that stage four new proteins (spots 6, 7, 9, 10) appeared in addition to three proteins (spots 4, 5, 8) already detected in the 36-h HCl-treated heterozygote. Therefore, with the exception of spot 15, the pattern of appearance and disappearance of proteins in the 48-h HCl-treated heterozygote was eventually comparable with that in the 48-h homozygote (compare Fig. 3b with Fig. 2g). From these results, it is clear that only one protein (spot 15) out of the eight heterozygote-specific proteins behaves according to the genotype irrespective of the developmental nature, but the remaining seven proteins behave according to the developmental nature. This finding suggests that spot 15 must be the *pnd*$^+$ gene-specific protein, but the remaining seven spots must be synthesized in parallel with the initiation of diapause.

To examine the effect of the diapause factor on the patterns of proteins synthesized in the heterozygote, heterozygous embryos which had not been exposed to the diapause factor during oogenesis were prepared by removing the suboesophageal ganglion from homozygous *pnd* female moths (see Materials and methods) and mating them with wild-type males. The resulting heterozygotes failed to enter diapause, even though they carried a copy of the *pnd*$^+$ gene. The eggs produced in this experiment will be called heterozygous non-diapause eggs to distinguish them from heterozygotes of the diapause type. Examples of the patterns of [^{35}S]methionine-labelled proteins extracted from the heterozygous non-diapause eggs are shown in Fig. 4. There are no appreciable differences in the patterns of proteins

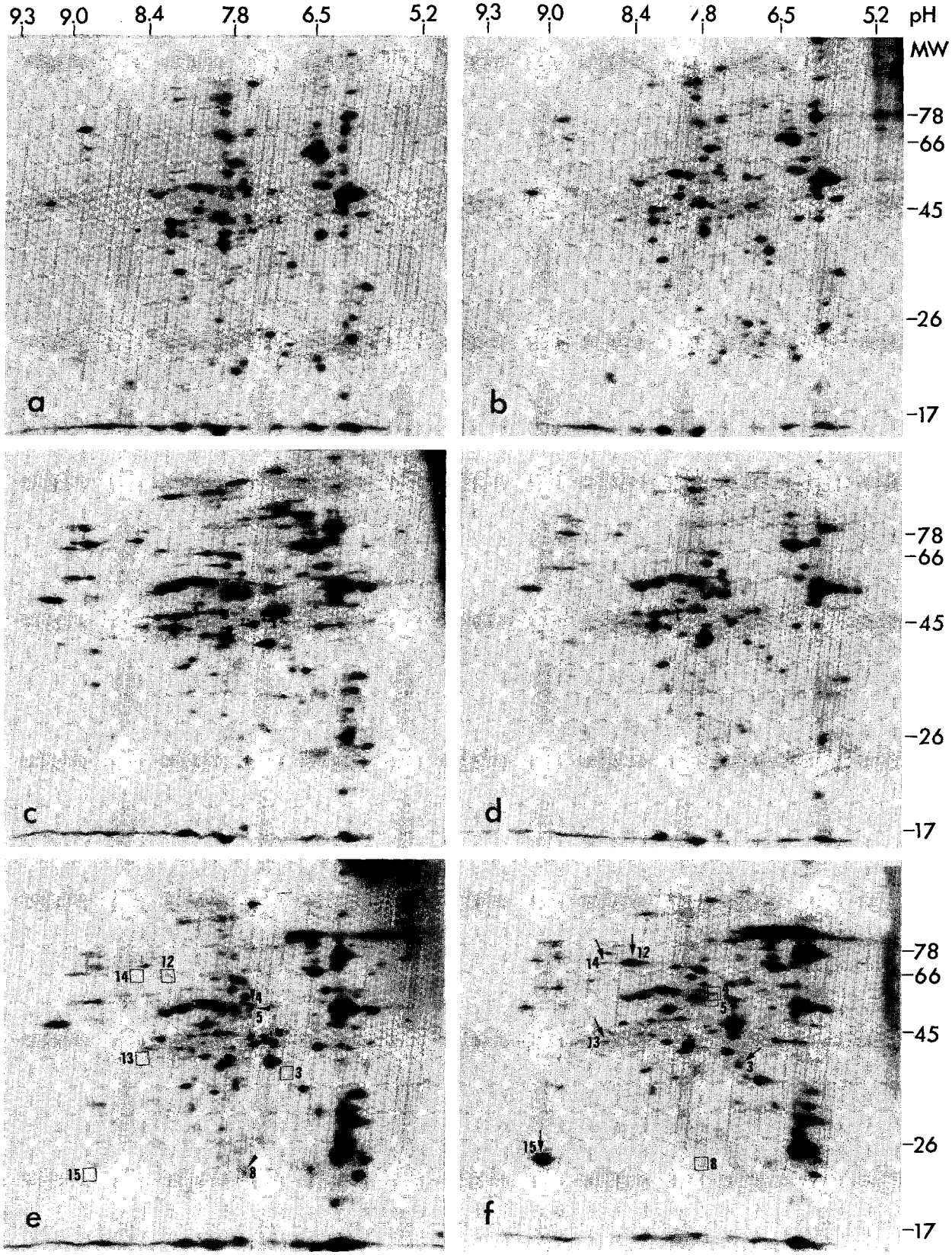
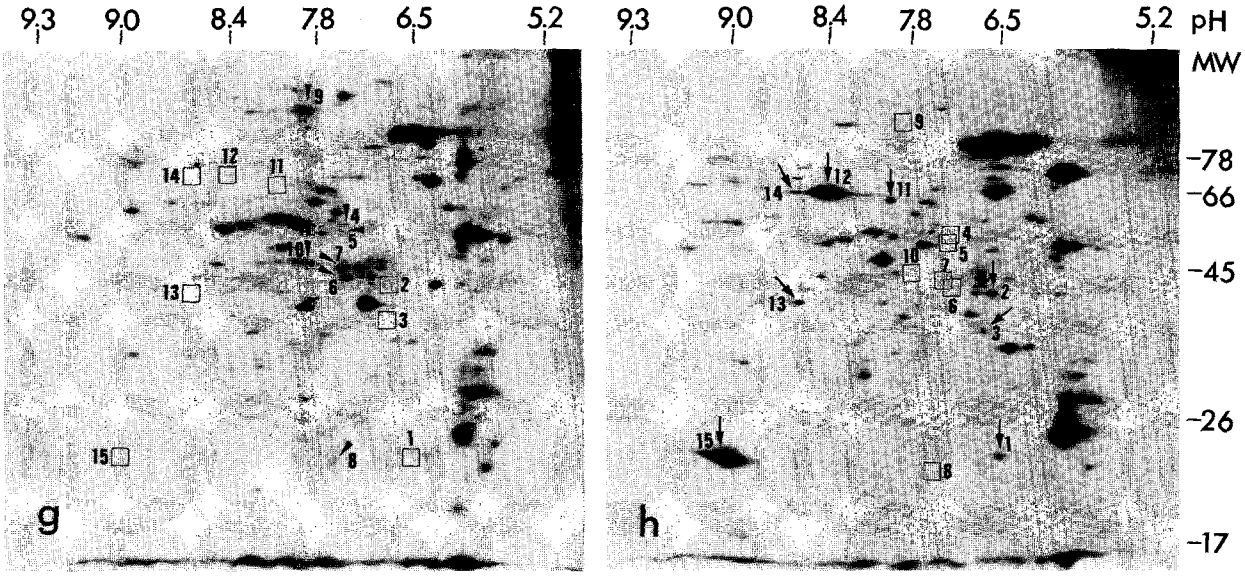


Fig. 2a–h. Fluorographs of two-dimensional gels of the proteins extracted from homozygote and heterozygote of the *pnd* mutant at various stages of early development. **a** 12-h homozygote; **b** 12-h heterozygote; **c** 24-h homozygote; **d** 24-h heterozygote; **e** 36-h homozygote; **f** 36-h heterozygote; **g** 48-h homozygote; **h** 48-h heterozygote. Eggs were injected with 20 nl [^{35}S]methionine and then incubated for 3 h at 25 °C. The [^{35}S]methionine-labelled proteins were separated, first by non-equilibrium pH gradient electrophoresis in the horizontal dimension, from right to left, and then by electrophoresis in the presence of sodium dodecyl sulphate (SDS) from *top to bottom*. TCA-precipitable cpm applied to the gels and the duration of X-ray film exposure are as follows: **a–g** 100,000 cpm, 14 days; **h** 58,000, 24 days. The pH gradient and the molecular weight scale (shown $\times 10^{-3}$) were determined as described in Materials and methods. *Arrows* indicate the proteins specific to the heterozygote. *Arrowheads* indicate the proteins whose presence can serve as an indicator of embryonic development (see text). *Squares* indicate expected locations on the gels used in comparisons. The patterns were highly reproducible



synthesized in either the heterozygous non-diapause eggs (Fig. 4a, b) or heterozygous diapause eggs (Fig. 2b, d) until 24 h after oviposition. At 36 h after oviposition, the heterozygous non-diapause eggs synthesized only spot 15 among the heterozygote-specific proteins, but they failed to synthesize a set of other heterozygote-specific proteins (compare Fig. 4c with Fig. 2f). At that stage, except for spot 15, the pattern of appearance and disappearance of the proteins was comparable with that of the homozygote (compare Fig. 4c with Fig. 2e). These facts suggest that all of the heterozygote-specific proteins except spot 15 behave as non-diapause type. The same trend of alteration as seen in the 36-h embryo was also observed at 48 h after oviposition as well (compare Fig. 4d with Fig. 2g, h).

From these results, it is clear that only spot 15 among the heterozygote-specific proteins is synthesized in the heterozygote at least from 36 h after oviposition, independent of whether or not the eggs have been exposed to the diapause factor during oogenesis, while the other heterozygote-specific proteins are synthesized only after the heterozygote has been exposed to the diapause factor during their formation. This finding suggests again that the behaviour of spot 15 depends exclusively on the embryonic genome, but the others depend exclusively on the developmental nature of the embryo.

Discussion

As shown in Fig. 1, the difference in the rate of protein synthesis associated with the difference in the embryonic

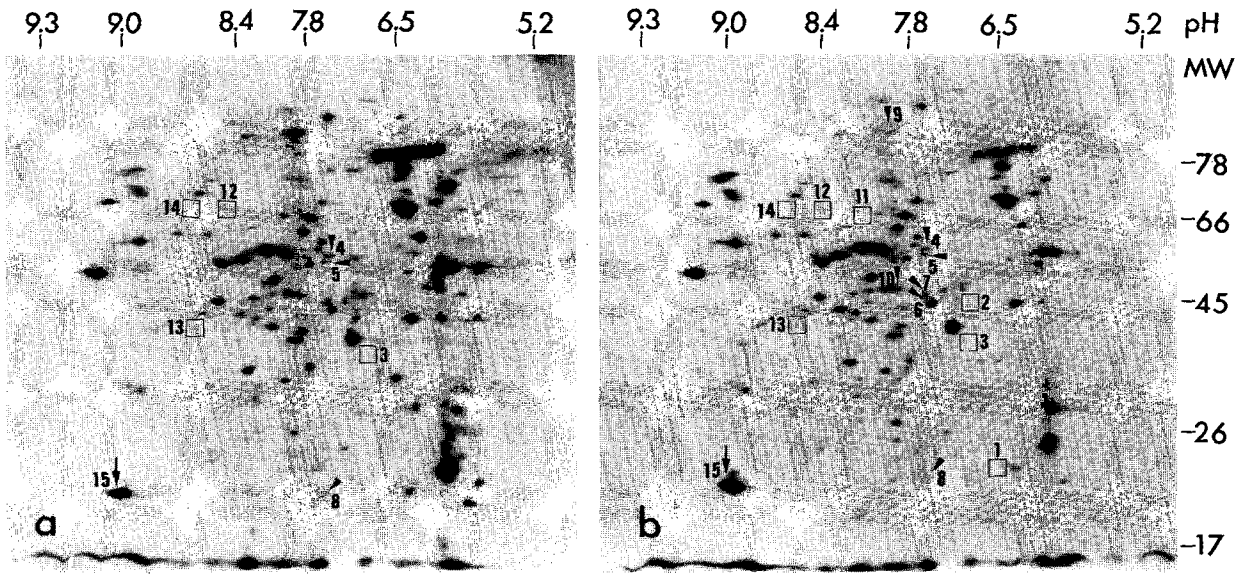


Fig. 3a, b. Fluorographs of two-dimensional gels of the proteins extracted from HCl-treated heterozygotes. The 24-h heterozygotes (prospective diapause eggs) were treated with HCl solution (see Materials and methods), and the HCl-treated eggs were injected with [^{35}S]methionine at 36 h (**a**) or 48 h (**b**) after oviposition. The [^{35}S]methionine-labelled proteins were analysed by two-dimensional gel electrophoresis. TCA-precipitable radioactivity applied to the gels and the duration of X-ray film exposure are 100,000 cpm and 14 days, respectively. The patterns were highly reproducible. Other details are as described in the legend to Fig. 2

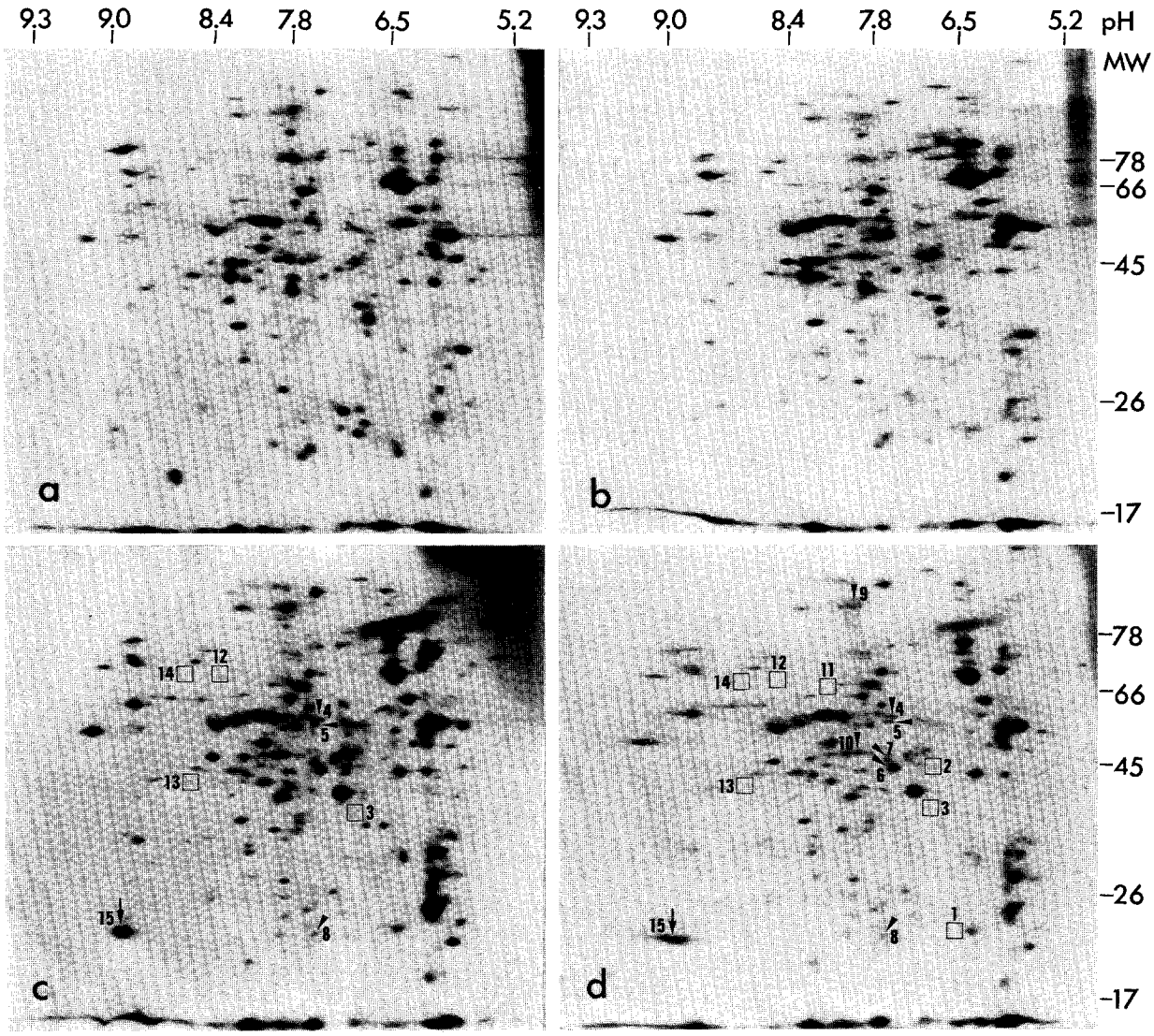


Fig. 4a–d. Fluorographs of two-dimensional gels of the proteins extracted from the heterozygous non-diapause eggs. The homozygous *pnd* female whose suboesophageal ganglion was removed just after larval-pupal ecdysis was mated to the wild-type male. The resulting heterozygotes (heterozygous non-diapause eggs) were injected with [^{35}S]methionine at different stages of embryogenesis. The [^{35}S]methionine-labelled proteins were analysed by two-dimensional electrophoresis. **a** 12-h eggs; **b** 24 h eggs; **c** 36-h eggs; **d** 48-h eggs. TCA-precipitable cpm applied to the gels and the duration of X-ray film exposure are as follows: **a, b** 85,000 cpm, 16 days; **c, d** 100,000 cpm, 14 days. The patterns were highly reproducible. Other details are as described in the legend to Fig. 2

genotype is apparent at 36 h after oviposition. This result suggests that the paternal *pnd*$^+$ gene has become active in the heterozygous embryo at least in the middle stage of gastrulation, and its product has participated in reducing the rate of protein synthesis. The finding as to the time of action of the *pnd*$^+$ gene agrees with results obtained on changing patterns of anaerobic carbohydrate metabolism (Sonobe 1984), amino acid pools (Sonobe and Okada 1984), and the rate of DNA synthesis (Sonobe et al. 1986) between the two types of eggs. Therefore, it was expected that a *pnd*$^+$ gene-specific product would be detected in the heterozygote at 36 h after oviposition (middle stage of gastrulation). In our preliminary experiments, we could not detect appreciable differences between the homozygote and heterozygote of the *pnd* mutant at 36–48 h after oviposition on two-dimensional gels stained with Coomassie brilliant blue R-250 (Sonobe et al., unpublished work). Thus [^{35}S]methionine labelled-proteins were analysed by means of two-dimensional gel electrophoresis. As shown in Fig. 2 (e–h), eight proteins specific to the heterozygous embryos were detected 36–48 h after oviposition, even though the rate of protein synthesis in the heterozygote decreased remarkably at that period as compared with the homozygote (Fig. 1). Among these eight heterozygote-specific proteins, it is strongly suggested that only one protein (no. 15), which is basic (approximately pH 9.0) and which has a molecular weight of approximately 22,000, must be the product of the *pnd*$^+$ gene.

Three main pieces of evidence support this hypothesis. First, the *pnd* mutant has been kept by outbreeding to pure strain Chinese 124, and *pnd* progeny was segregated by mating between F_1silkworms heterozygous for the *pnd* allele. The frequent outbreedings to Chinese 124 make it certain that the two strains remained isogenic except for the mutant gene *pnd*. In these two isogenic strains, spot 15 appeared exclusively in the heterozygote carrying the pat-

ternal pnd^+ gene, but not in the recessive homozygote (Fig. 2e–h). The time of its appearance does not conflict with the time at which biochemical changes associated with the initiation of diapause begin in the heterozygote (Fig. 1; Sonobe 1984; Sonobe and Okada 1984; Sonobe et al. 1986). Second, only spot 15 among the eight heterozygote-specific proteins was synthesized according to the embryonic genome without being affected by the artificial alteration of development (Fig. 3). This finding was confirmed by the changing patterns of proteins synthesized by heterozygous non-diapause eggs (Fig. 4c, d). These results exclude the possibility that spot 15 was synthesized as a secondary consequence of diapause initiation. Third, genetic data on the *pnd* mutant have indicated that the *pnd* gene is a single recessive gene (Yamamoto et al. 1978). This suggests that the pnd^+ gene may code for a single functional protein. The results of our present experiments are consistent with a deficiency in one specific protein in the recessive homozygote. Consistent results have also been obtained by two-dimensional gel electrophoresis, in which isoelectric focusing gels (O'Farrell 1975) were used in the first dimension in order to analyse more acidic proteins, or in which other percentages of acrylamide were used in the second dimension slab gel in order to cover the other ranges of molecular weight (Sonobe and Odake, unpublished work).

In the heterozygous non-diapause eggs, it was demonstrated that the putative pnd^+ gene-specific protein is also synthesized in eggs which have not been exposed to the diapause factor during oogenesis (Fig. 4c, d). This finding does not contradict our observation that in the wild-type silkworm the putative pnd^+ gene-specific protein appears not only in diapause eggs, but also in non-diapause eggs 36 h after oviposition (unpublished data). From these results, we can conclude that the putative pnd^+ gene-specific protein is synthesized independent of the action of the diapause factor.

In the wild-type silkworm, all of the heterozygote-specific proteins except for the putative pnd^+ gene-specific protein were also observed more in the diapause eggs than in those that were non-diapause (unpublished data). This finding is consistent with the result that, except for the putative pnd^+ gene-specific protein, the appearance of the heterozygote-specific proteins parallels the initiation of diapause in heterozygous *pnd* embryos (Figs. 2–4). Therefore, these proteins must be a useful indicator of diapause initiation, and we will refer henceforth to these proteins as diapause-associated proteins.

The role of the diapause-associated proteins is obscure at present, but it is conceivable that they may control some of the rapid biochemical changes associated with the initiation of diapause, such as onset of characteristic metabolism concurrent with a decrease in oxygen uptake (Sonobe 1984; Sonobe and Okada 1984), a decrease in the rate of DNA synthesis (Sonobe et al. 1986), and a decrease in the rate of protein synthesis (Fig. 1). If that is the case, there is a possibility that, in eggs which have been exposed to the diapause factor during oogenesis, the putative pnd^+ gene-specific protein may stimulate the synthesis of diapause-associated proteins, but in eggs not so exposed the putative pnd^+ gene-specific protein may not stimulate such a synthesis. In other words, the diapause factor may be involved in the process by which the pnd^+ gene-specific protein exerts its function at the level of protein systhesis. Further experiments are necessary to clarify the biochemical roles of this protein and the diapause-associated proteins produced at the onset of diapause.

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