

Analysis of nuclear proteins from silk glands of *Bombyx mori*

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Abstract. A gentle method for the isolation of nuclei from developing silk glands of *Bombyx mori* has been standardized. The nuclei, whether isolated or directly visualized *in situ* within the silk glands, exhibit complex morphology. The nuclei occupy almost the entire volume of the gigantic silk gland cells. Although the isolated nuclei still retain their ramified morphology, being polyploid they are fragile and often become fragmented. The histone and low-salt-extractable proteins from nuclei isolated from the middle and posterior silk glands on different days of the fourth and fifth instars of larval development have been analysed. The histones did not show any stage- or tissue-specific variations whereas the low-salt-extractable proteins showed some developmental stage specific variation. Using the antibody raised against one such protein, its absence in the early stage of development has been confirmed by Western blotting techniques. This developmental stage specific protein may be functionally linked to some activities responsible for boosting up the production of silk or silk-related proteins during the fifth instar of larval development.

Keywords. Nuclei isolation; ramified nuclei; developmental stage specific proteins; histones; silk gland nuclei; silk gland proteins.

Introduction

The silkworm *Bombyx mori* possesses a pair of long, tubular organs called the silk glands which are divided into anatomically and functionally distinct regions (Suzuki, 1977). The silk glands produce the major classes of silk proteins. Fibroin, the silk fibre protein is synthesized in the posterior silk gland (PSG) (Couple *et al.*, 1983; Kimura *et al.*, 1985), and sericins, a group of adhesive proteins that coat the fibroin are produced in the middle silk gland (MSG) (Ishikawa and Suzuki, 1985). The genes that encode these proteins are actively expressed in a developmental stage specific manner mainly during the fifth instar of larval development (Suzuki, 1977; Prudhomme and Couple, 1979).

The silk glands of *B. mori* are fully formed at the end of embryonic development (Goldsmith and Kafatos, 1984) and no further cell divisions take place afterwards. However, the cells grow much larger in size as development progresses. The nuclei of silk gland cells undergo dramatic changes in morphology in the course of larval maturation. During larval development, DNA synthesis in the middle and the posterior silk glands continues without cell division. The DNA content of these polyploid nuclei increases by about 2×10^5 times over that of the diploid nuclei (Gage, 1974; Tashiro *et al.*, 1968). Many rounds of endormtotic DNA replication (Suzuki *et al.*, 1972; Suzuki, 1977; Tazima, 1978; Perdrix-Gillot, 1979) occur during

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Abbreviations used: PSG, Posterior silk, gland; MSG, middle silk gland; PMSF, phenylmethylsulphonyl fluoride; SDS, sodium dodecyl sulphate; CBB, Coomassie brilliant blue; PBS, phosphate-buffered saline.

the last 3 instars: an average of 18-19 doublings in the posterior, 19-20 in the middle, and 13 in the anterior silk gland. Due to polyploidization the nuclei of silk gland cells become progressively ramified. In fact, at the middle of the fifth instar an extremely ramified nucleus spreads all over within the cell (Akai, 1983). Fragility, the highly lobate nature of the ramified nuclei and the presence within the cells of a large amount of silk proteins, which are either easily transformed into insoluble masses or coprecipitate with nuclei, pose major difficulties in preparing pure nuclei (Suzuki and Giza, 1976). It requires special care to isolate nuclei of such unusual morphology. We have standardized a simple procedure to isolate the nuclei in sufficient purity. The pure preparations of nuclei were used to analyse the histone- and low-salt-extractable proteins. Electrophoresis of the extracted proteins from middle and posterior silk gland nuclei on different days of the fourth and fifth instars was carried out to examine any tissue and developmental stage specific variations.

Materials and methods

The silk worm *Bombyx mori* NB₄D₂ strain was used for all the experiments. The biochemicals and reagents were from the Sigma Chemical Company, St Louis, Missouri, USA.

The PSG and MSG from larvae in the late third instar and on all days of the fourth and fifth instars were examined. The excised glands were briefly washed in ice-cold KCl (100 mM), rapidly frozen in liquid nitrogen and stored at -90°C.

Staining of nuclei located within the silk glands

The frozen glands of the late third or fourth instar were thawed in glycerine-Hanks solution (1:1, v/v) (Ichimura *et al.*, 1985) and of the fifth instar in Hanks solution (0.14 M NaCl, 5.4 mM KCl, 0.8 mM MgSO₄, 0.9 mM CaCl₂, 3 mM KH₂PO₄, 3 mM Na₂HPO₄ and 0.1 % glucose, pH 6.1). The thawed glands were incubated with a few drops of collagenase (Worthington Biochemicals, 0.5 units/ml) in Hanks solution, pH 7.2, for 5 h at 37°C. The glands were then stained with orcein (0.4% in 90% ethanol), rinsed once with 45% acetic acid, mounted on a glass slide under a cover slip in 50% glycerol, and observed under bright field. The photographs were taken using a Zeiss transmitted light photomicroscope. A similar set of glands treated with collagenase was stained with acridine orange (10 µg/ml in PBS) for 15 min at room temperature, washed 4-5 times with PBS and mounted on a glass slide in 50% glycerol in PBS. Fluorescence micrographs were taken (Adams and Kamensky, 1971) using a Zeiss epifluorescence condenser III RS D 7082 fluorescence microscope.

Isolation of nuclei

The frozen PSG and MSG of all days of the fourth and fifth instars were thawed in a small amount of Hanks solution, crushed very gently with a pestle in a glass mortar and filtered through a nylon mesh (pore size 1 mm). The central columnar fibroin, fragments of basement membrane, and other large pieces of contaminants

(e.g. tracheae) remain on the nylon mesh. In the case of PSG the samples were kept at -10°C for about 4h to denature and coagulate the fibroin prior to filtration. The cytoplasm present in the filtrate was removed by repeated suspension and decantation in Hanks solution until the crude nuclei were left as a sediment in clear Hanks solution. Nuclei were treated with 0.5% Nonidet P-40 (NP-40) and centrifuged at 300 g for 2 min in a swinging bucket rotor. The pellet was suspended in TMK buffer (10mM Tris-HCl, pH 7.8, 3mM MgCl_2 , 150 mM KCl, 0.5 mM dithiothreitol) containing NP-40, stirred for 30 min and filtered through a very fine nylon mesh (pore size 0.1 mm). This crude preparation was applied on the top of a step gradient comprising of 40% (10 ml) and 80% (3 ml) Percoll in TMK buffer (Kondo *et al.*, 1987) and centrifuged at 600 g for 20 min. The nuclei located at the interface were again applied on a second gradient consisting of 50% and 80% Percoll. The nuclei from the interface were checked for purity and the presence of any cytoplasmic material by fluorescence microscopy after staining with 0.01% acridine orange in PBS.

The DNA and RNA contents of the preparation were quantitated by the diphenylamine reaction (Burton, 1956) and the orcinol reaction (Ceriotti, 1955) respectively. Protein was estimated according to Lowry *et al.* (1951).

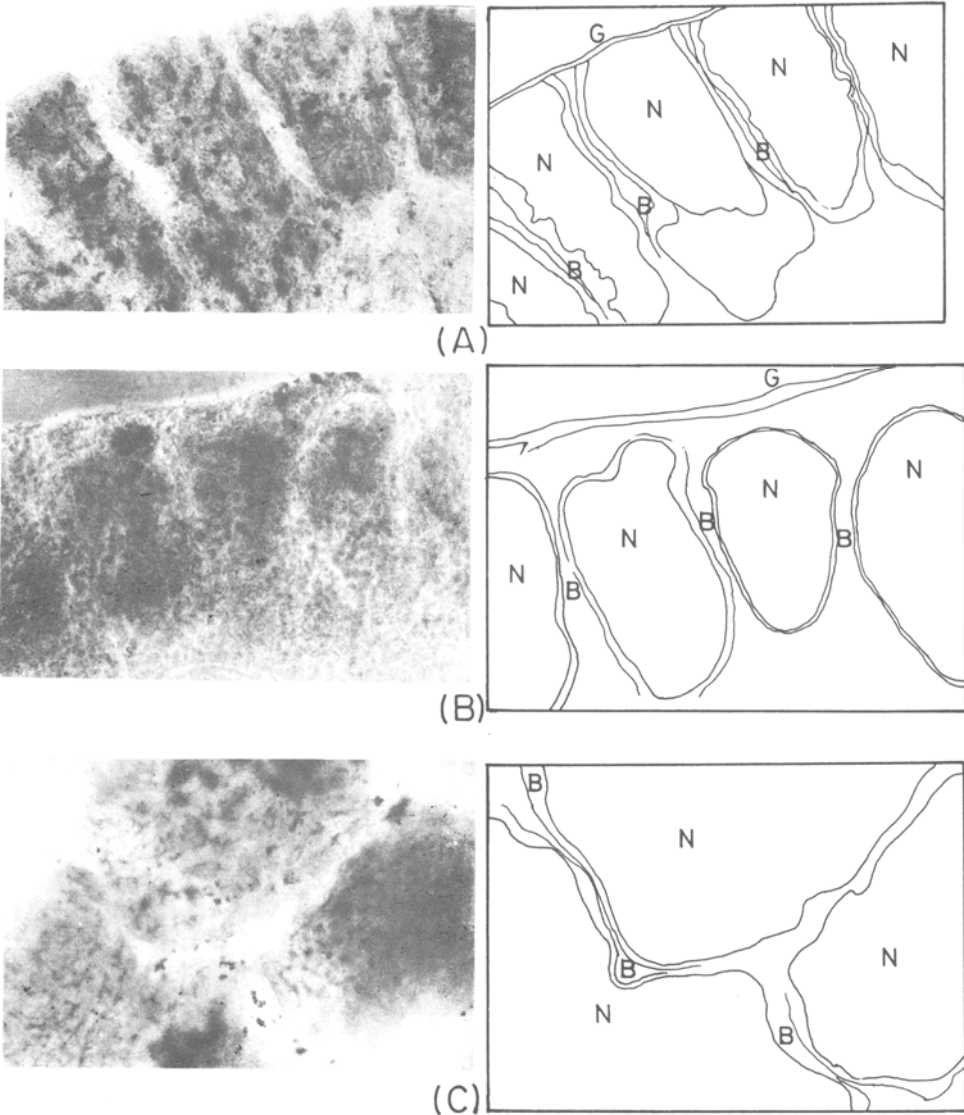
Analysis of the nuclear proteins

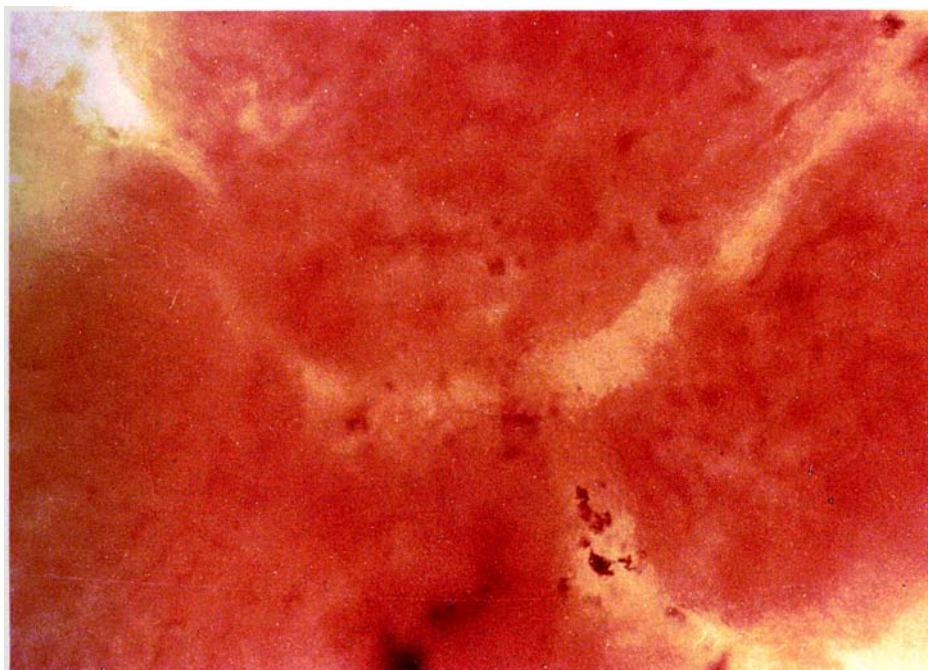
The isolated nuclei from MSG and PSG of different days of the fourth and fifth instars were treated twice with low concentration of salt (0.25 M NaCl) and 0.1 mM phenylmethylsulphonyl fluoride (PMSF) to extract the non-histone proteins. This was followed by extraction of histone proteins using dilute acid (0.25 N HCl). The histone and low-salt-extractable proteins were analysed by electrophoresis on Polyacrylamide gels containing sodium dodecyl sulphate (SDS). The histone proteins (extracted with 0.25 N HCl) were separated on 12.5% SDS-polyacrylamide gels and stained with 0.25% Coomassie brilliant blue-G (CBB) in 40% methanol and 10% acetic acid. The 0.25 M NaCl extracted proteins were separated on 9% SDS-polyacrylamide gels. These gels were first stained with 0.25% CBB and then by the silver staining procedure (Morrissey, 1981).

Antibodies to a specific protein

In order to raise antibodies against a single protein appearing in a developmental stage specific manner, preparative SDS-polyacrylamide slab gel electrophoresis was carried out on 9% gels. About 3–4 mg of total low-salt-extractable proteins from fifth instar silk gland nuclei were loaded onto the gel. Electrophoresis of nuclear proteins was carried out at constant voltage (80–100 V). The gel was fixed in 5% acetic acid when the run was completed. Longitudinal strips (1 cm wide) in the direction of the run were cut from either side of the gel and stained with 0.02% CBB. The stained strips were aligned with the edges of unstained gel and the region corresponding to the specific band was cut separately and used for antibody production. The gel slice was initially kept in 10% ethanol to remove the SDS, dialysed against phosphate-buffered saline (PBS), homogenized in Potter-Elvehjem homogenizer and suspended in PBS. The suspension was emulsified with Freund's complete adjuvant and the emulsion injected intramuscularly into a rabbit. Sub-

sequently 3 injections (in the presence of Freund's incomplete adjuvant) were administered at weekly intervals. A fifth injection was given subcutaneously and finally a booster injection using the solubilized protein eluted from acrylamide gel slice. Blood (10–15 ml) was collected through the marginal ear vein 5–6 days after the final injection and allowed to clot overnight in a refrigerator. The serum was clarified by centrifugation (3250 g, 10 min) and was tested for the presence of antibody by the Ouchterlony double diffusion method as well as the more sensitive Western blotting technique.





(D)

Figure 1. Visualization of nuclei within the silk glands. Bright field micrographs of orcein-stained PSG cell nuclei of (A) fourth day of third instar, (B) third day of fourth instar, and (C) fourth day of fifth instar. (D) Same as C, shown in colour. Line drawings are also provided for clarity. Magnification is the same in all the photographs. N, nucleus; B, cell boundary; G, silk gland boundary.

Western blotting

The method described by Towbin *et al.* (1979) was used with some modifications. The low salt (0.25 M NaCl) extracted proteins from nuclei isolated from fourth and fifth instars silk glands were separated on a 9% Polyacrylamide gel under denaturing conditions. The samples were included in duplicate in two separate portions and after the run the gel was cut longitudinally into halves. One half was stained with CBB and the other was electrophoretically blotted for 12–16 h onto nitrocellulose filter. The transferred proteins on the nitrocellulose filter were probed with antiserum raised against the 50 kDa protein seen in extracts from nuclei of fifth instar glands. The filter was first soaked in PBS containing 2–3% BSA and 0.3% Tween 20, incubated for 2–3 h at room temperature and washed 3 times (20 min each) with PBS containing 0.05% Tween 20, on a shaker. The filter was then incubated for 2 h at 37°C with the antiserum (diluted 1:10 in PBS, containing 0.05% Tween 20). Subsequently the filter was washed with PBS-Tween for 1 h with 3 changes of buffer and incubated for 1 h with goat antirabbit IgG-horseradish peroxidase (HRPO) conjugate. The filter was washed thrice in PBS-Tween and once in PBS and then incubated for 10 min in 10 ml of citrate buffer containing diaminobenzidine (10 mg), CoCl_2 (0.1 ml of 1% solution) and 7.5 μl of 30% H_2O_2

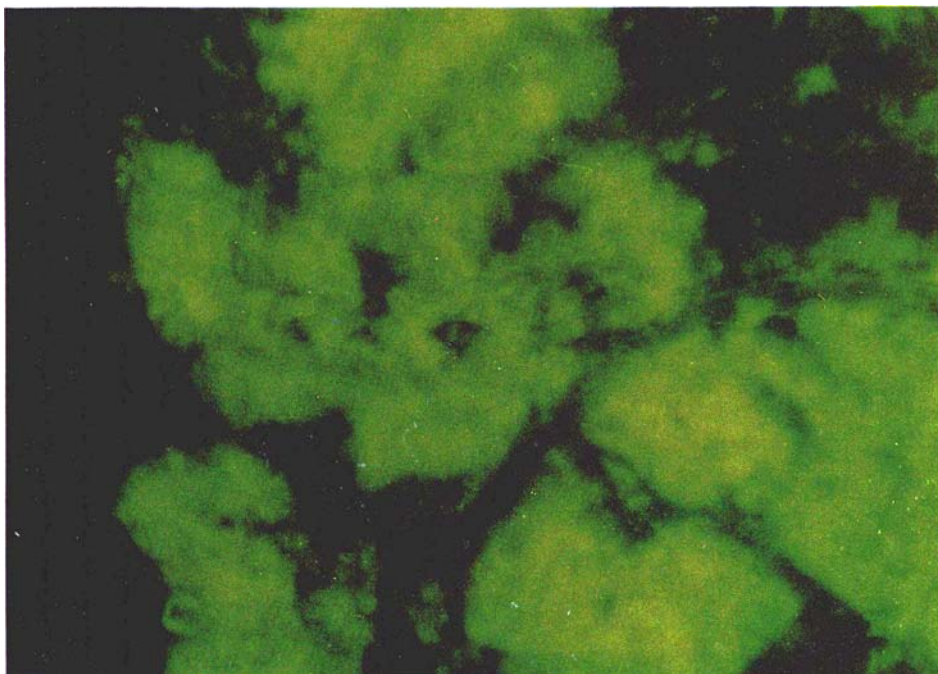


Figure 3. Isolated nuclei. The nuclei isolated from fifth instar PSG were stained with acridine orange and examined under fluorescence microscope. (A) The yellow to green fluorescence represents the nuclear material. Absence of red fluorescence confirms that there is no cytoplasmic contamination. N. Nucleus.

to develop the colour reaction (Hsu and Soban, 1982). Appropriate controls with nonimmune serum were always included.

Results

Staining of nuclei within the silk glands

The middle and posterior silk glands of late third and all days of fourth and fifth instars were treated with collagenase to remove connective tissue material and directly stained with orcein or acridine orange. Figure 1 shows the bright field micrographs of orcein-stained nuclei within the silk gland at late third, fourth and fifth instars. The boundaries of the gigantic cells making up the silk glands and the nuclei nearly filling the entire volume of the cells though diffused, can be made out. For clarity line reproductions of the photographs are also provided. The nuclei are elongated in the direction of the long axis of the cells in third instar. Ramification of the nucleus starts in the last days of the third instar (figure 1A) and in the fourth instar many lobes stretch to form a long backbone (figure 1B).

The fluorescence micrographs of acridine orange stained nuclei within the silk gland at fourth and fifth instars are shown in figure 2. For comparison phase contrast micrographs of the same preparations are also presented. The silk gland

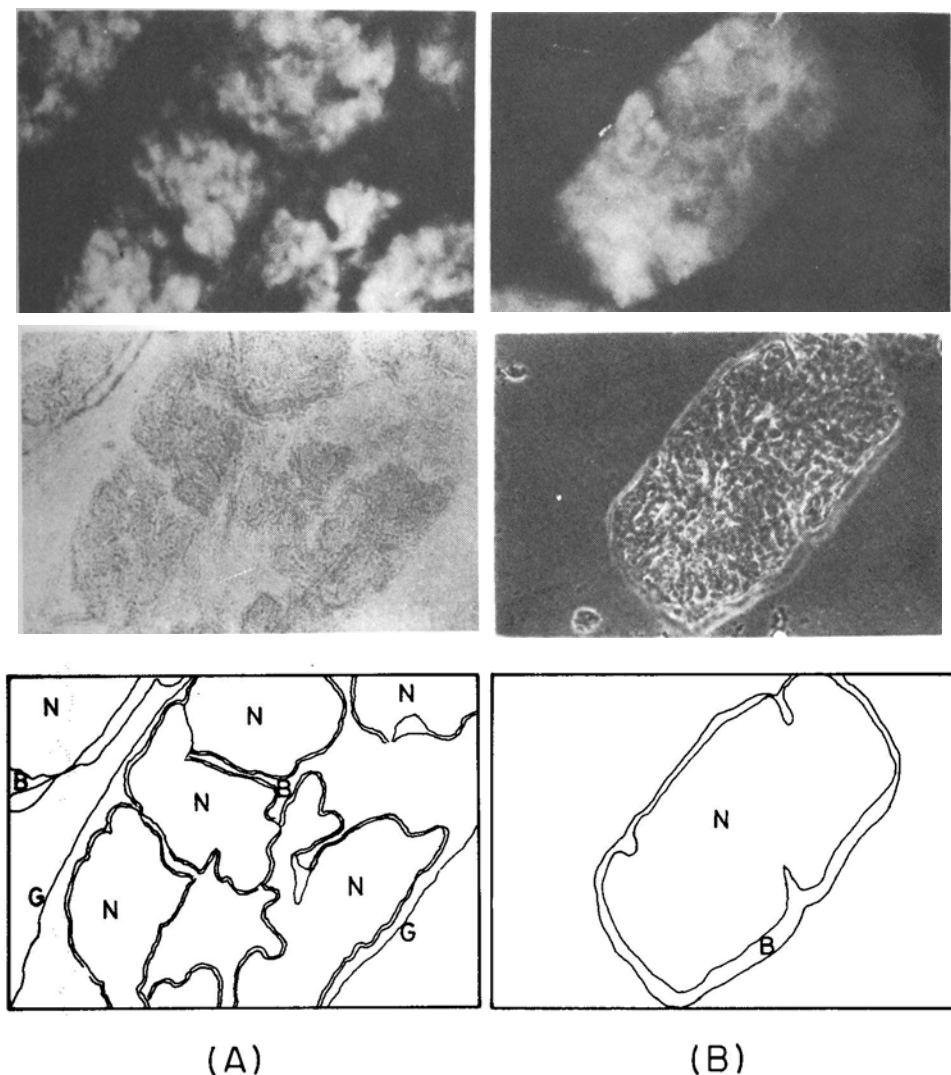


Figure 2. Fluorescence staining of nuclei within the silk gland. Fluorescence micrographs of the acridine orange-stained nuclei within the PSG: (A) fourth day of fourth instar and (B) fifth day of fifth instar. The corresponding phase contrast micrographs and line drawings are shown in lower rows of each column. N, Nucleus; B, cell boundary; G, silk gland boundary.

nuclei occupy almost the entire cell volume (compare fluorescence with corresponding phase contrast micrograph in figure 2B). The major structures visible in the glands are due to nuclei while the cell boundaries are not very distinct. The treatment of the glands with collagenase also makes the cell boundaries diffuse. However, the gigantic size and the increasing dimensions of the cells in development can be clearly made out (all the pictures were taken at the same magnification). The nuclei of the silk gland cells become progressively ramified. An extremely ramified nucleus spreads all over within the cell at the middle of the fifth instar.

Isolation of nuclei

The middle and posterior silk glands of different days of the fourth and fifth instars were excised for isolation of nuclei. During isolation, the nuclei get fragmented to some extent even under extremely mild conditions. Although fragments of varying sizes were observed, they retained the ramified morphology. The fluorescence micrographs of the isolated nuclei after acridine orange staining show negligible cytoplasmic contamination, as evidenced by the absence of red fluorescence (figure 3). The nuclei isolated by Kondo *et al.* (1987) had substantial cytoplasmic contamination. Although the basic techniques were similar (see discussion) the method we have utilized yielded better preparations of nuclei. The weight ratio of DNA/RNA/histone proteins/low-salt-extractable proteins was found to be approximately 1:1:1:2.5, substantiating the purity of the nuclear preparation.

Analysis of the nuclear proteins

The acid-soluble proteins extracted by 0.25 N HCl from nuclei of MSG and PSG of different days of fourth and fifth instar larvae were analysed on 12.5% SDS-polyacrylamide gels. The electrophoresis pattern in figure 4 demonstrates the existence of the 4 core histones and histone H1. There were additional protein bands, presumably other species of histones or modified histones as well as nonhistone proteins present in the acid-extracted samples from both the tissues and

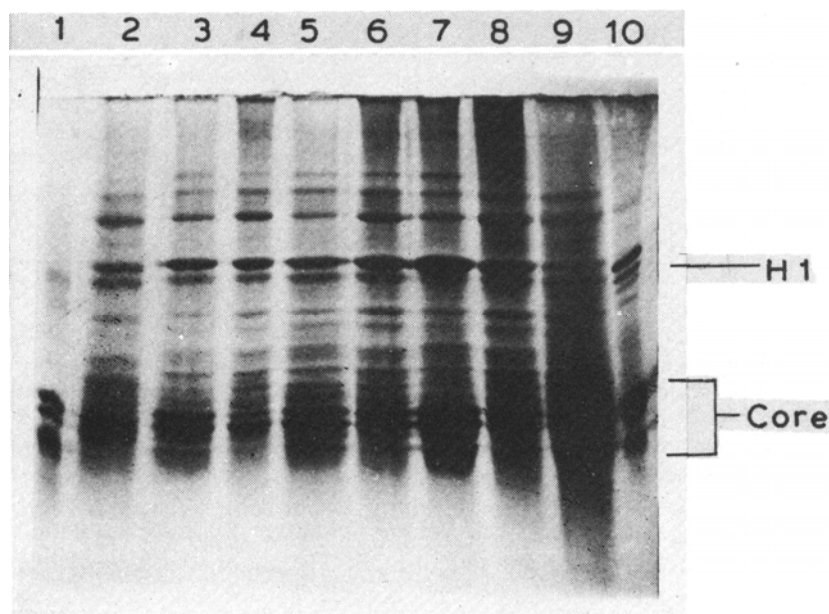


Figure 4. Histones from silk gland nuclei. The histone proteins extracted with 0.25 N HCl from silk gland nuclei of different stages were subjected to electrophoresis on 12.5% SDS-polyacrylamide gels. The gels were stained with CBB. Lanes 1 and 10, standard histone markers; lanes 2-5, fourth instar samples, in the order MSG and PSG of second day and MSG and PSG of fourth day respectively; lanes 6-9, fifth instar samples, in the order MSG and PSG of second day and MSG and PSG of fifth day.

at all the stages. By and large the histone composition was similar in MSG and PSG nuclei at all the stages. The additional bands of proteins seen in the histone gels were present irrespective of whether the previous extraction was carried out with 0.25 M or 0.35 M NaCl. On the other hand, prior treatment with 0.35 M NaCl removed the histone HI. Therefore we resorted to using 0.25 M NaCl in all the subsequent experiments.

The overall pattern of distribution of acid extractable proteins in PSG and MSG nuclei was similar.

The electrophoresis pattern of the 0.25 M NaCl extracted proteins on SDS-polyacrylamide gel is presented in figure 5. A large number of proteins were visualized and some of them were negatively stained with silver when present in excessive amounts. Nevertheless some development stage specific variations are obvious in the protein banding pattern. For instance, a protein of about 50 kDa was present in samples of all days of the fifth instar in both MSG and PSG (arrow in figure 5). This band was not traceable in either of the tissues in any fourth instar

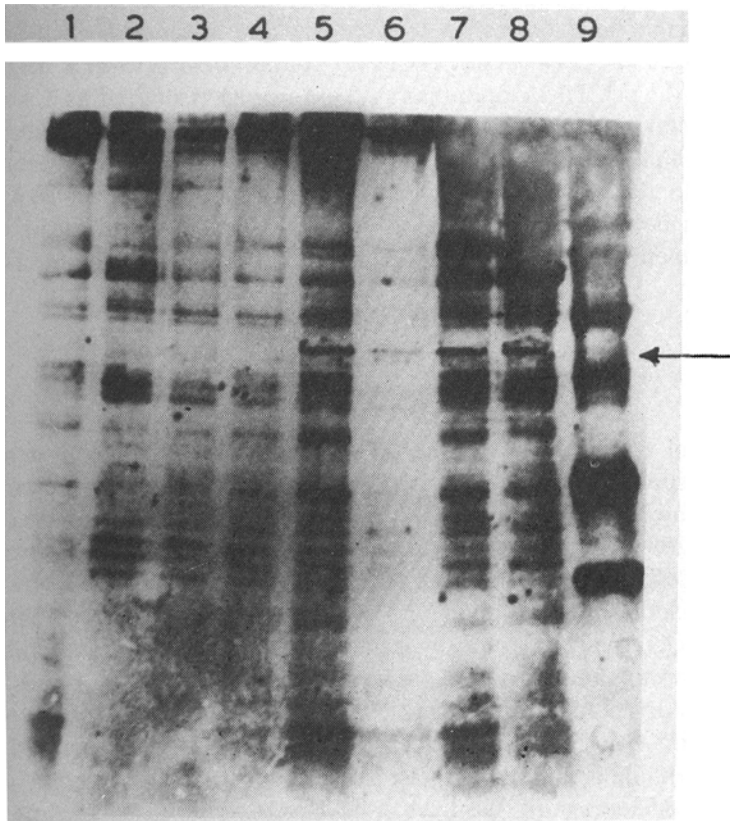


Figure 5. Low-salt-extractable proteins of silk gland nuclei. Proteins of silk gland nuclei extracted with 0.25 M NaCl were subjected to electrophoresis on SDS-polyacrylamide gels (9%). The gel was stained with CBB and then by silver staining. Lanes 1–4, fourth instar samples in the order MSG and PSG of second day and MSG and PSG of fourth day respectively; lanes 5–8, fifth instar samples in the order second day MSG and first day PSG, and MSG and PSG of fifth day; lane 9, standard protein markers. The arrow indicates the position of the band present only in fifth instar lanes.

sample. Similar variations were seen for some other proteins. The absence of the 50 kDa protein in the fourth instar samples was consistently observed in all the preparations.

Antibodies to the developmental stage specific protein

The 50 kDa protein which was detected in fifth instar silk gland cell nuclei but was conspicuously absent in the fourth instar silk gland nuclei was used for immunizing a rabbit for the production of antibodies. The rabbit serum, collected after the administration of even the booster dose of the specific protein, did not give the precipitin band in the Ouchterlony immunodiffusion test. The failure to demonstrate the presence of specific antibody in the serum by this test led us to the more sensitive Western blotting method. The results of the immunoblotting are presented in figure 6, which shows the specific protein revealed by antibody. This result confirms the presence of specific antibody in the serum. No bands were seen in control blots probed with nonimmune serum. The blot probed with immune serum shows two adjoining bands (figure 6), migrating close to each other. Although one single band of protein was cut out and injected to elicit antibodies, the heterogeneity could have arisen owing to contamination from a neighbouring protein band in the gel. The contaminating band can be visualized as a weak signal in the fourth instar lane also. Most importantly, however, the lane containing the fourth instar sample of nuclear proteins did not show the immunoreactive band corresponding to the 50 kDa protein, confirming its absence. The 50 kDa band could be seen in both MSG and PSG of the fifth instar. Thus, this protein appears to be a developmental stage specific protein present in both the tissue only during the fifth instar.

Discussion

The silk gland of *Bombyx mori* has served as a convenient model system for the study of tissue specific and developmental stage specific gene expression (Suzuki, 1977; Prudhomme and Couble, 1979). The MSG and PSG are made up of approximately 255 and 520 cells respectively (Goldsmith and Kafatos, 1984). During larval development, the cells continue to grow larger in size without division. The nuclei also do not divide; however, the DNA replication continues. As a result, the nuclei grow enormously large in size and become ramified. It has proved to be a formidable task to isolate intact nuclei from the silk gland. Isolation of nearly satisfactory preparations of nuclei from the silk glands have been recently reported (Ichimura *et al.*, 1985; Kondo *et al.*, 1987). Although the method we have developed here for the isolation of nuclei is similar to that of Kondo *et al.* (1987), there are some differences: (i) We have used Hanks solution as starting working medium against TMK buffer used by Kondo *et al.* (1987). (ii) We have repeatedly decanted the nuclear preparation with Hanks solution until the crude nuclei were left as a sediment in clear solution. This step is very crucial, (iii) We have employed NP-40 in place of Triton X-100.

Fluorescence microscopy of the nuclei after staining with acridine orange and their macromolecular composition demonstrated their purity. Our nuclear prepa-

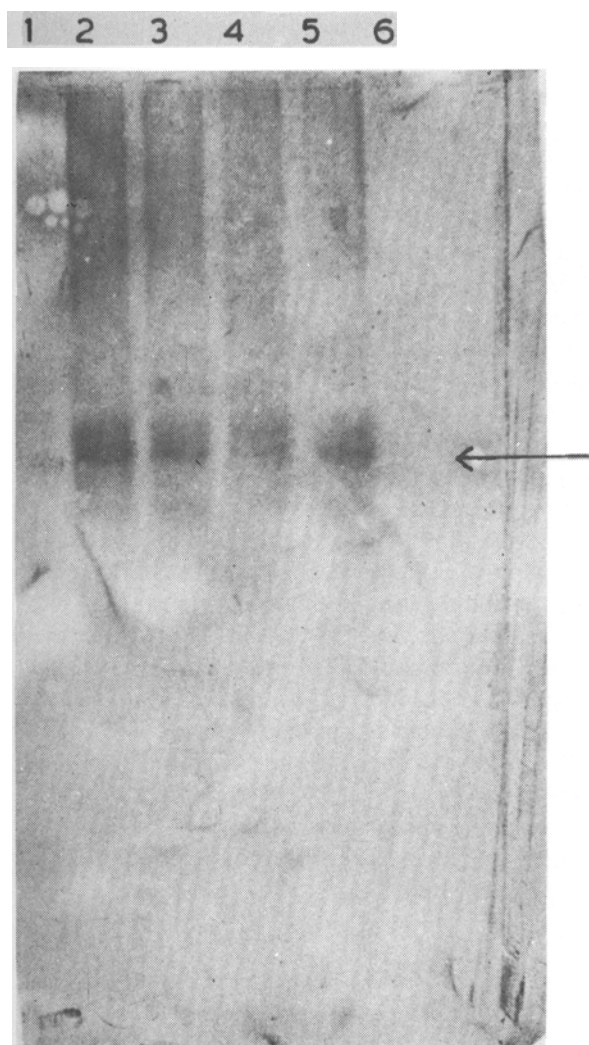


Figure 6. Western blot analysis. Antibodies raised against a single protein band (shown in figure 5) was used to probe the low-salt-extracted proteins from silk gland nuclei. The detection system used was the colour reaction catalysed by HRPO coupled to goat antirabbit IgG. Lane 1, fourth instar fourth day sample; lanes 2–5, fifth instar samples in the order MSG and PSG on third day and MSG and PSG on fifth day respectively; lane 6, standard marker protein. Arrow indicates the position of the 50 kDa protein band.

rations were clean and were devoid of cytoplasmic contamination. However, the fragility of the ramified nuclei was evident even in our preparations.

It was of interest to investigate the origin and the molecular mechanism of the formation of the higher order structure of the giant ramified nuclei and the chromatin in the nuclei of silk gland cells of *B. mori*. The MSG and PSG of different days of third, fourth and fifth instar, when treated with collagenase and stained with dyes (orcein or acridine orange) showed the ramified morphology of the cell nuclei. The ramification of the nucleus starts from the late third instar and

due to progressive ramification the nucleus occupies almost the entire cell volume by the middle of the fifth instar.

The silk glands of *B. mori* are highly differentiated to produce the silk proteins. The production of silk occurs in a tissue-specific and development stage specific manner. From the reported literature on the control of gene expression in analogous systems, it is evident that the formation of appropriate chromatin structures is necessary for the control of *in vivo* transcription. The association and distribution of proteins on the chromatin are expected to show differences depending on the state—expressing or non-expressing—of the genome. The histone protein pattern in MSG or PSG did not show any differences at any stage. The presence of a 50 kDa nonhistone protein, however, in both MSG and PSG nuclei only during the fifth instar was conspicuous; this protein was absent in the fourth instar. Since it is known that massive silk production starts only towards the end of the fifth instar, the appearance of this protein during the fifth instar may be of significance. The synthesis of silk involves the production of fibroin and sericins, and other accessory proteins, as well as the necessary gearing-up of the system. Since the 50 kDa protein was found in both MSG and PSG, it may not be directly related to fibroin synthesis but rather may be involved in the regulation of expression of any or all of fifth instar protein(s) including the silk-related proteins.

In order to assign a specific function to this 50 kDa protein in silk glands, we have begun by taking an immunological approach to detect it in nuclear extracts. Although this protein was not highly antigenic, we could demonstrate the presence of specific antibodies in serum of immunized rabbit by the sensitive Western blotting method. We propose to use this antibody to isolate and purify the specific protein(s) from the fifth instar glands by the immunoaffinity procedure. The relation, if any, between the appearance of the 50 kDa protein in a developmental stage specific manner in both MSG and PSG and the synthesis of silk proteins is not evident at this point of time.

Acknowledgements

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