ORGANIZATION OF THE CHORION GENES OF *BOMBYX MORI*, A MULTIGENE FAMILY. III. DETAILED MARKER COMPOSITION OF THREE GENE CLUSTERS

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Manuscript received November 8, 1979
Revised copy received May 16, 1980

ABSTRACT

The chorion patterns produced by progeny from crosses that were used to define the linked gene clusters, *Ch 1*, *Ch 2* and *Ch 3* (strain C108 *vs.* strain Ascoli), were examined by two-dimensional gel electrophoresis (isoelectric focusing *vs.* SDS/urea). Approximately 60 proteins were assigned to four previously defined chorion classes, A, B, C and H_c, believed to represent the products of related genes, on the basis of size and relative cysteine content. All strain-specific markers showed co-dominance in the F_1, indicating the likelihood that they are not products of post-translational modification. Twenty-seven Ascoli markers co-segregated in testcross progeny, and none of the resolved proteins showed independent assortment, confirming their linkage to chromosome *2*. Two-dimensional screening of recombinants demonstrated that all three clusters contain Class A and B markers; H_c markers have been found only in *Ch 1* and *Ch 2*; whereas, mapped C markers were confined to *Ch 3*. This indicates that chorion clusters are heterogeneous with respect to the markers they contain.

THE silkmoth chorion consists of 50 to 100 different polypeptides that are synthesized in a highly ordered developmental sequence by the follicular epithelium during the terminal stages of oogenesis (PAUL *et al.* 1972; NADEL and KAFATOS 1980). Similarities in the sizes, amino acid compositions (KAFATOS *et al.* 1977) and primary structure of chorion proteins (REGIER *et al.* 1978; RODAKIS 1978; KAFATOS *et al.* 1978; JONES *et al.* 1979) suggest that they are encoded by several families of related genes.

To gain an understanding of both the developmental regulation and the evolution of chorion genes, it is essential to know their detailed chromosomal arrangement. Using electrophoretic variants resolved by isoelectric focusing from inbred strains of *Bombyx mori* as genetic markers, we previously demonstrated that a large number of chorion genes are linked (GOLDSMITH and BASEHOAR 1978), and form at least three clusters on chromosome *2* (GOLDSMITH and CLERMONT-RATTNER 1979). We report here the detailed resolution of the set of mapped chorion markers from testcrosses between strains C108 and Ascoli (GOLDSMITH and CLERMONT-RATTNER 1979) by two-dimensional gel electrophoresis and the

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assignment of these markers to four defined protein classes. Our results confirm earlier findings of genetic co-dominance and indicate that chorion gene clusters are heterogeneous with respect to the classes of markers they contain.

MATERIALS AND METHODS

Silkworm stocks and rearing: Strains C108 and Ascoli were obtained from Y. TAZIMA and A. MURAKAMI of the National Institute of Genetics, Misima, Japan, and reared on fresh mulberry leaves, as previously reported by GOLDSMITH and BASEHOAR (1978).

Solubilization and in vitro *labeling of chorion proteins:* Chorions were cleaned, solubilized and carboxamidomethylated as described by GOLDSMITH and BASEHOAR (1978). Samples to be labeled with ^{14}C-iodoacetamide were solubilized at a concentration of 3 to 4 chorions/10 μl (approximately 20 μg protein/ μl) in 8 M urea, 0.05 M dithiothreitol, 1.5 mM lysine, 1.5 mM EDTA and 0.05 M Tris-HCl, pH 9.0 at room temperature. To 5 μl of dissolved protein we added 10 μl of a solution containing 4 M urea, 0.02 M iodoacetamide, 0.6 M Tris pH 8.4 and 2.5–5 μCi ^{14}C-iodoacetamide (Amersham, 13 to 26 mCi/mM) in subdued light. After 5 to 10 min, a half-volume of 0.42 M iodoacetamide in 1.2 M Tris-HCl, pH 8.4 was added; the reaction was terminated in 10 to 15 min by the addition of 3 μl β-mercaptoethanol per 100 μl final volume. Urea and nonradioactive iodoacetamide were purified as described elsewhere (GOLDSMITH and BASEHOAR 1978).

Two-dimensional gel electrophoresis: Samples were resolved by isoelectric focusing, followed by separation in the second dimension on slab gels containing sodium dodecyl sulfate and urea (GOLDSMITH *et al.* 1979). Mixed samples contained 45 to 60 μg nonradioactive protein and 4 to 8 ug radioactive protein. After electrophoresis, gels were fixed, stained and photographed as reported by GOLDSMITH *et al.* (1979), soaked in 1 to 3% glycerol for 30 min and dried on Whatman 3 MM filter paper, using a homemade vacuum suction apparatus. Autoradiographic exposures were carried out for 1 to 6 weeks with Kodak X-omat R film.

RESULTS

Parental protein patterns: Two-dimensional chorion protein patterns from parental strains C108 and Ascoli are shown in Figure 1. Approximately 60 major spots are visible in each strain by staining (left) compared with about 30 bands by isoelectric focusing alone (GOLDSMITH and BASEHOAR 1978). Proteins were assigned to three previously defined molecular weight classes that are characteristic of *B. mori*, designated A, B and C in order of increasing size, and a fourth class greatly enriched in cysteine, the H_c proteins (KAFATOS *et al.* 1977; NADEL and KAFATOS 1980). The A and B proteins are well separated in our gel system. The division between the B and C components was based on a slight separation in relative molecular weights and the presence of more basic proteins in the presumptive C's. In general, the mobilities of these spots corresponded well with the assignment of C proteins from other strains (NADEL and KAFATOS 1980). Finally, we identified H_c proteins by their relatively high specific activities in autoradiograms after *in vitro* labeling with ^{14}C-iodoacetamide (Figure 1, right), which reacts selectively with cysteine residues. For ease of identification, proteins of a particular strain were numbered consecutively within each of these classes from basic to acidic isoelectric points, high to low molecular weights. Thus, spots that originated in different strains, but had identical electrophoretic mobilities, were not always assigned the same numbers. This is in accordance with the

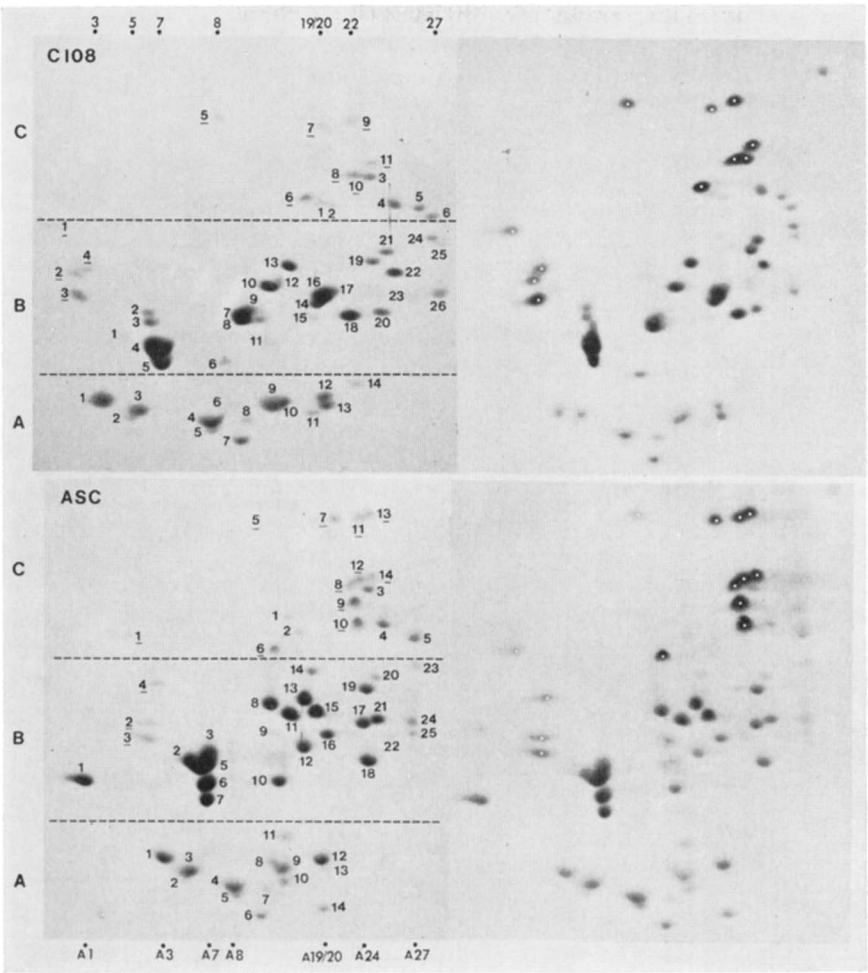

FIGURE 1.—Two-dimensional patterns of chorion proteins from C108 and Ascoli strains. Isoelectric focusing is in the horizontal dimension (basic to the left); SDS/urea is in the vertical dimension. All consistently resolved spots are numbered within specified protein classes. A, B and C proteins are separated by dotted lines; H_c proteins are underlined or marked with white dots. Approximate positions of major isoelectric focusing bands are indicated across the top (C108) and bottom (Ascoli). Left, Coomassie blue stain; right, autoradiograms of ^{14}C-carboxamidomethylated samples.

possibility that they might be products of nonidentical structural genes. Minor spots detectable only by labeling were not included, nor were a small number of proteins that fell outside the pH range of 4 to 5.5, since they were not screened routinely in our genetic analyses.

To determine which spots were strain-specific, we also carried out separations on mixed samples containing nonradioactive chorion from one strain and small quantities of *in vitro* labeled chorion from the other. Nonradioactive components were detected by staining with Coomassie Brilliant Blue, labeled proteins by

autoradiography. Thus, proteins with very similar mobilities could be distinguished in the same gel.

The increase in resolution revealed that electrophoretic differences that appeared to be quantitative by isoelectric focusing along (GOLDSMITH and BASEHOAR 1978) were actually produced by qualitatively different proteins. For example, the marked difference in intensity of isoelectric focusing band A7 *vs.* band 7 was produced by three major B proteins (2, 3 and 5) specific to the Ascoli strain, not by different amounts of proteins common to both strains. Similarly, three B proteins (14, 16 and 17) in C108 were lacking in Ascoli and accounted for the relatively high intensity of band 19/20.

Although the patterns of the two strains were broadly similar, the number, intensity and distribution of proteins differed considerably (Table 1). Class A and C proteins were nearly identical, with the majority of differences occurring in minor components. The greatest amount of polymorphism occurred in the B proteins, which constitute the predominant group (KAFATOS *et al.* 1977; NADEL and KAFATOS 1980). While the total number of B components was essentially the same (25 in Ascoli *vs.* 26 in C108), only two major spots and four minor spots had identical mobilities and relative intensities. Particularly striking was the

TABLE 1

Correlation of C108 and Ascoli two-dimensional chorion patterns

A. Proteins common to both strains*

Protein class	Spot number
A	**1(1)**, 2(2), **3(3)**, **4(4)**, 5(5), **7(6)**, 8(7), **9**(9)
B	**4(6)**, **5(7)**, 9(9), 23(22), 24(23), 26(25)
C	**3(3)**, **4(4)**, **5(5)**
H_c	1(1), 2(2), 4(4), 5(5), 7(7), 8(8), 9(11), 10(12), 11(14)

B. Strain-specific proteins

C108‡	
A	6, 10, 11, **12**†, **13**, 14
B	6, **7**, **8**, **10**, 11, **13**, **14**, **16**, 17, **18**, **19**†, **20**, **21**, **22**†, 25
C	1, 2
H_c	2, 6
Ascoli	
A	8, 10, 11, **12**, 13†, 14
B	**1**, **2**, **3**, **5**, **8**, **10**, **11**, **12**, **13**, 14, **15**, **16**, **17**, **18**, **19**†, 20†, **21**, 24
C	1, 2
H_c	3, 6, 9, 10, 13

* C108 numbers are listed first and corresponding Ascoli numbers are listed in parentheses.
† Spots with identical electrophoretic mobilities but different intensities. Not scored in testcross progeny.
‡ C108 class B spots 2, 3 and 15 were not observable in testcross progeny because of overlap with major Ascoli-specific spots.
Major spots are listed in boldface.

presence of four extra major B proteins and three added H_c proteins in the Ascoli strain. This diversity made it impossible to determine which spots were allelic without further information regarding their amino acid sequences or genetic fine structure.

Co-dominance: The co-dominant expression of all strain-specific proteins was demonstrated in the two-dimensional chorion patterns produced by crossing C108 and Ascoli (Figure 2). Thus, the F_1 was qualitatively identical to samples obtained by mixing proteins from both strains. This confirmed our previous findings upon screening by isoelectric focusing alone. Further, it appeared that many of the major B spots were present at half the parental levels (for example 8, 11, 13 and 15 from Ascoli and 10, 13, 14 and 16 from C108), as expected from a simple gene dose relationship. Two Ascoli class B proteins, 17 and 21, were found in only two of the four samples screened. Their relative pI's suggest that they probably correspond to isoelectric focusing markers A24 and A26, which were reported to be heterozygous in the Ascoli stock (GOLDSMITH and BASEHOAR 1978).

Linkage of four classes of chorion structural genes: We had previously shown that progeny produced by testcrossing the F_1 (C108/Ascoli) with C108 segregated into two predominant phenotypic classes: Class F, whose chorion pattern was identical to the F_1, and Class P, identical to C108 (GOLDSMITH and BASEHOAR 1978; GOLDSMITH and CLERMONT-RATTNER 1979). These studies demonstrated the linkage of 13 isoelectric focusing markers from the Ascoli strain. To determine the protein class that these markers represented, we rescreened chorions produced by progeny selected from three pair matings of F_1 males with C108 females. Again, all of the progeny were identical to the appropriate parental protein patterns (Figure 3). Thus, we were able to score a total of 27 co-segregating

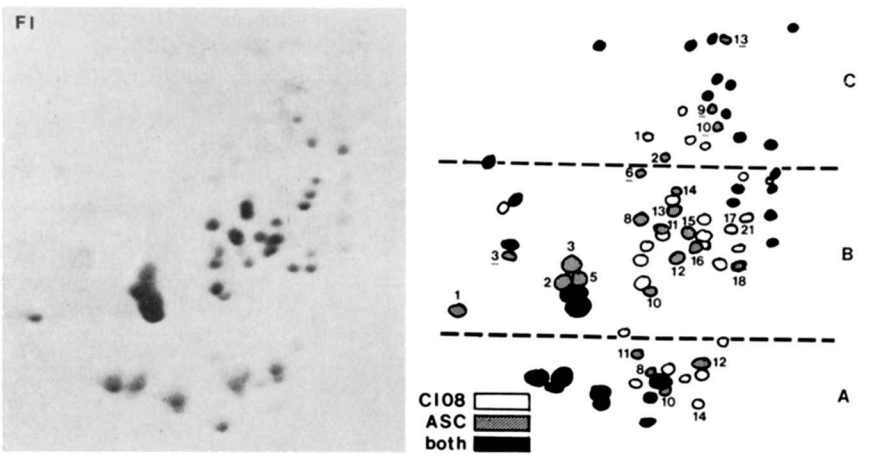

FIGURE 2.—Two-dimensional pattern of chorion proteins from F_1 progeny. The parental strain of each protein is indicated on the diagram. Numbered spots were mapped in subsequent crosses; H_c proteins are underlined. Class B spots 17 and 21 (dotted) are heterozygous in the Ascoli strain.

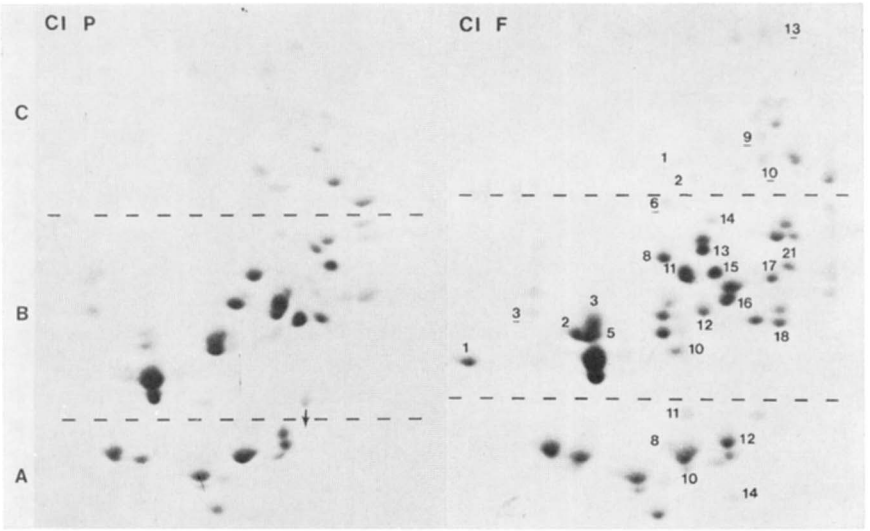

FIGURE 3.—Two-dimensional patterns of chorion proteins from Class P and Class F progeny. Numbered markers are Ascoli-specific; all others are C108-specific or common to both strains. H_c proteins are underlined. Arrow indicates the usual gel position of a protein assigned to Class A.

Ascoli-specific markers in Class F samples. These included proteins in all four chorion classes (Table 1A).

We took extreme care to analyze the patterns of any samples that showed anomalous behavior on isoelectric focusing alone. In all, we examined 40 samples by two-dimensional electrophoresis. Twelve Class P and 12 Class F samples were scored for all protein classes, while the remaining 16 samples were scored for B, C and H_c proteins. It is noteworthy that even with the increased gel resolution, no markers showed independent assortment with respect to the Class F and Class P phenotypes. This provides strong evidence for the linkage of genes encoding the four major groups of chorion proteins.

Detailed composition of chorion gene clusters: We also obtained 11 testcross progeny that produced three recombinant chorion patterns on isoelectric focusing gels, each expressing a different subset of the previously scored Ascoli markers (GOLDSMITH and CLERMONT-RATTNER 1979). Two appeared to be reciprocals, designated classes R1a and R1b. The third, Class R2, carried all of the R1b markers, but only two of the seven possible isoelectric focusing bands characteristic of Class R1a. These patterns were used to define three chorion gene clusters, *Ch 1*, *Ch 2* and *Ch 3* (Figure 4).

Two-dimensional analysis of chorions from all of the recombinant progeny confirmed and extended these findings (Figure 5). All progeny assigned to a particular phenotypic class on the basis of isoelectric focusing patterns were identical even with higher resolution. Moreover, Classes R1a and R1b were indeed reciprocals: R1a expressed 19 of the possible Ascoli markers, while R1b produced the remaining eight spots (Table 2). Finally, as expected, Class R2 contained

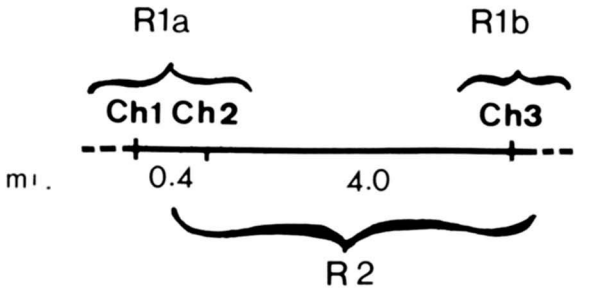

FIGURE 4.—Relationship between chorion recombinants and mapped chorion gene clusters *Ch1*, *Ch2* and *Ch3*. Phenotypic classes R1a and R1b represent reciprocal crossovers between *Ch2* and *Ch3*. Together, these recombinants indicate all markers mapped to chromosome *2*. Class R2 represents a crossover between *Ch1* and *Ch2*. One progeny containing all markers from *Ch2* and *Ch3* was recovered.

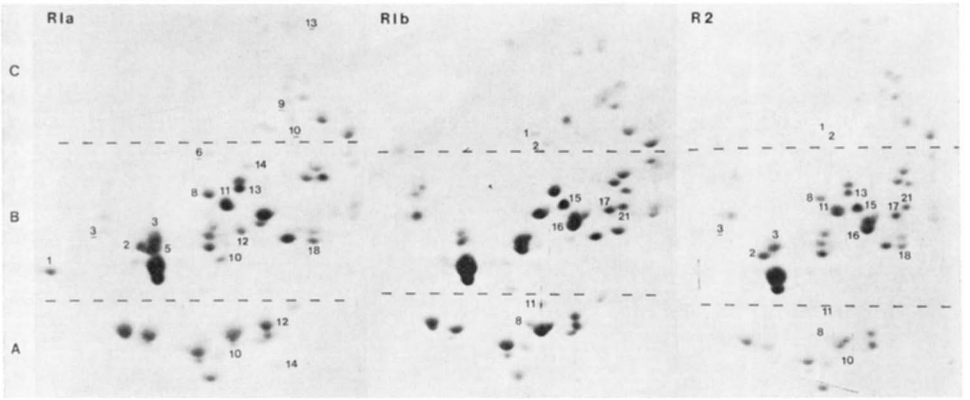

FIGURE 5.—Two dimensional patterns of chorion proteins from recombinant progeny classes R1a, R1b and R2. Numbered markers are Ascoli-specific; all others are C108-specific or common to both strains. H_c proteins are underlined.

TABLE 2

Ascoli marker composition in recombinant progeny

Protein class	Progeny class		
	R1a	R1b	R2
A	10, 12, 14	8, 11	8, 10, 11
B	1, 2, 3, 5, 8, 10, 11, 12, 13, 14, 18	15, 16, 17, 21	2, 3, 8, 11, 13, 15, 16, 17, 18, 21
C	—	1, 2	1, 2
H_c	3, 6, 9, 10, 13	—	3

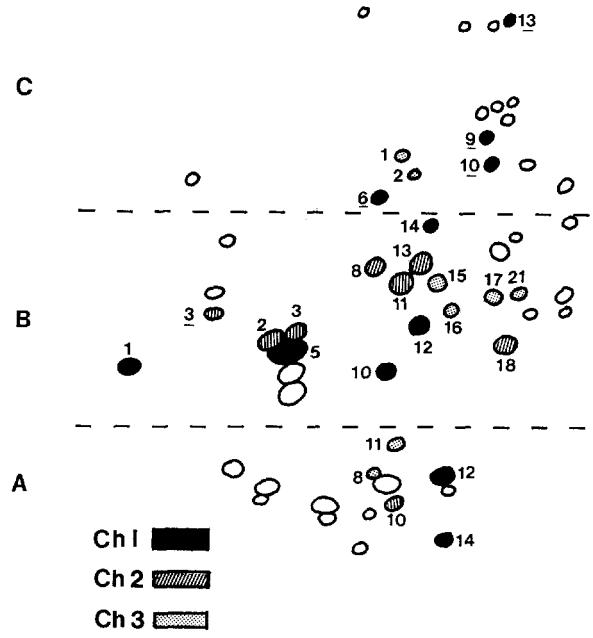

FIGURE 6.—Detailed marker composition of *Ch1*, *Ch2* and *Ch3*. Depicted is the two-dimensional chorion pattern from the Ascoli strain (see Figure 1). Numbered spots were assigned to one of three gene clusters as indicated; H_c markers are underlined.

all of the R1b markers, plus eight additional R1a-specific components. Thus, markers encoding both A and B proteins were found in all three clusters; however, H_c markers were found only in *Ch 1* and *Ch 2*, while C markers were confined to *Ch 3* (Figure 6).

DISCUSSION

We have demonstrated that approximately half of the chorion components from a single strain of *B. mori* resolved by two-dimensional electrophoresis behave as linked genetic markers. Their co-dominant, dose-dependent expression constitutes strong evidence that these electrophoretic variants reflect mutations in chorion structural genes rather than strain-specific differences in protein modifier genes. Further, all of these markers have been observed in hybrids with other inbred strains of *B. mori* (unpublished). These results contrast with the observation that most modifier genes show simple dominance; thus, genetic background has a profound effect on the additivity of specific electrophoretic markers in hybrids (FINNERTY and JOHNSON 1979). Our conclusion is reinforced by the ability to resolve these markers into three separate loci by genetic recombination.

Since the electrophoretic variants used in this study were chosen at random from many available inbred silkworm races, it seems likely that the majority of *B. mori* chorion genes will prove to be linked to chromosome *2*. However, a number of mutations that affect external chorion morphology have been reported, two of which are known to be unlinked: *Se*, white-sided egg, (CHIKUSHI

1972) and *mgr*, mottled grey egg, (H. DOIRA, personal communication). It is possible that these genes code for defective structural proteins that interfere with assembly of the complex, highly organized ultrastructure of the chorion (KAFATOS *et al.* 1977; GOLDSMITH *et al.* 1978; MAZUR, REGIER and KAFATOS 1980). Alternatively, these loci may encode processing or regulatory functions that are essential for normal chorion morphogenesis or affect the relative abundance of chorion constituents. The latter appears to be the case for the mutation Gr^{col} (*2*–6.9), which causes slower rates of secretion and subsequent intracellular degradation of chorion proteins that are electrophoretically indistinguishable from wild type (NADEL *et al.* 1980). Mutations that perturb chorion ultrastructure and are accompanied by reduced amounts of specific chorion proteins have also been observed in *Drosophila melanogaster* (DIGAN *et al.* 1979). One of these, ocelliless, maps in the vicinity of the affected structural genes (SPRADLING, WARING and MAHOWALD 1979).

The markers investigated in this study include representatives of four defined protein classes that have a number of striking biochemical similarities, including distinctive and unusual amino acid compositions, acidic pI's and narrow molecular weight ranges (KAFATOS *et al.* 1977; G. RODAKIS, personal communication). These properties suggest that *B. mori* chorion proteins are encoded by related genes. Indeed, common ancestry has been demonstrated for the Class A and B proteins of the Saturniid moth, *Antheraea polyphemus*. Thus, amino acid sequencing studies (REGIER *et al.* 1978; RODAKIS 1978) and the characterization of cloned cDNA's synthesized by reverse transcriptase from chorion mRNA (KAFATOS *et al.* 1978; SIM *et al.* 1979; JONES *et al.* 1979) show that A and B genes comprise two distinct but related families. Moreover, antibodies made to fully denatured, purified A, B, and C proteins from *A. polyphemus* cross-react extensively with chorion proteins from *B. mori* (J. R. HUNSLEY, personal communication). Although this is not rigorous proof for sequence homology, it is a strong indication that an analogous situation exists in the domesticated silkworm.

Each of the chorion clusters found thus far on chromosome *2* contains A and B markers, as well as markers from the C or H_c classes. Whether members of a particular chorion class are grouped together or interspersed within the clusters must be determined by fine-structure analysis, which is now possible by application of recombinant DNA techniques. Using this approach, JONES and KAFATOS (1980) recently reported the isolation of two genomic DNA fragments from *A. polyphemus* carrying alternating copies of A and B genes. The sequences on a given fragment were shown to be expressed at a particular period in choriogenesis (SIM *et al.* 1979), suggesting the possibility that chorion genes are arranged in coordinately expressed units. In this regard, it is intriguing that the *B. mori* C and H_c markers mapped in this study are found in the clusters most distal to one another. Class C proteins are synthesized first in choriogenesis, while the H_c proteins are the last to be translated (KAFATOS *et al.* 1977; NADEL and KAFATOS 1980; BOCK and GOLDSMITH in preparation; M. CHOTI, unpublished observations). We are presently carrying out experiments to determine whether the A and B markers also show developmental specificity as a function

of their map positions. Sea urchin histone genes appear to be organized along superficially similar lines, with interspersion of different gene families and transcription from different gene sets at defined developmental periods (KEDES 1979). *Xenopus laevis* and *X. borealis* also carry two clusters of 5S RNA genes that show differential developmental expression (FEDEROFF 1979).

The genetic basis for the peculiar variability in the number of major chorion components in different true-breeding silkworm lines may reflect differences in chorion gene number, levels of expression of a number of potentially "silent alleles," or both. In this respect, the chorion system is reminiscent of the mammalian histocompatibility genes, in which it is still not known whether the dramatic range and variety of haplotypes is attributable primarily to differences in structural or regulatory genes (KLEIN 1978). The likelihood that tandemly linked multigene families undergo rapid expansion and contraction by mechanisms involving unequal sister-chromatid exchange (SMITH 1973, 1976; TARTOF 1974; HOOD, CAMPBELL and ELGIN 1975; OHTA 1976, 1977, 1979) and unequal crossing over (SCHALET 1969; FRANKHAM, BRISCOE and NURTHEN 1978; KIMURA and OHTA 1979) argues for the the plausibility of both possibilities. Structural analysis of several A and B sequences of *A. polyphemus* demonstrates that these kinds of events have occurred within structural genes during evolution of the Saturniid chorion families (JONES *et al.* 1979). The unusual size polymorphism evident in the two-dimensional chorion patterns of purebred and hybrid lines suggests that large deletions or duplications have also occurred in *B. mori* chorion genes, perhaps as a result of similar mechanisms. (For example, for B proteins, in Figure 1, note Ascoli spots 3, 5, 6, 7 and C108 spots 4 and 5, 2 and 3; in Figure 2, compare Ascoli spots 11 and 13 with C 108 spots 10 and 13, respectively.) If unequal crossing over includes spacer sequences, it would not only affect the final number of functional chorion genes present in a recombinant chromosome, but it might also alter regulatory sites that carry information for initiation of transcription, RNA processing or developmental timing (FEDEROFF 1979). Further investigation of the chromosomal regions containing chorion genes at the DNA level will help resolve this issue.

Supported by National Science Foundation grant PCM 76–82450 to M. R. GOLDSMITH.

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