ORGANIZATION OF THE CHORION GENES OF *BOMBYX MORI*, A MULTIGENE FAMILY. I. EVIDENCE FOR LINKAGE TO CHROMOSOME *2*

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ABSTRACT

The chorion genes of silkmoths comprise a multigene family that codes for 50 or more highly specialized structural proteins found in the eggshell. A detailed study of the chromosomal organization of these genes was initiated, using inbred stocks of *Bombyx mori* as a source of electrophoretic variants for genetic markers. Chorion protein patterns were screened on thin-slab polyacrylamide isoelectric focusing gels. A wide range of polymorphism was observed between stocks. However, isoelectric focusing patterns obtained within a stock were nearly homogeneous, indicating that inbreeding has produced a high degree of homozygosis. Testcrosses were carried out to examine the linkage relationships between electrophoretic markers in four inbred stocks. One race (C108) was selected as a standard against which to compare the inheritance of the variants found in the other three stocks. Chorion markers behaved like codominant Mendelian traits in F_1 crosses. A total of 15 out of 16 C108 markers cosegregated in subsequent testcrosses, indicating that they are linked. These genes were mapped to the second chromosome, using markers *Gr* and *Y*.

THE existence of operons in which a set of genes that code for functionally related products come under coordinate control has long been investigated in in eukaryotes. To date, evidence for operon-like gene clusters (DAVIS 1975; GREER and FINK 1975), or even *cis*-acting regulatory elements, is limited to relatively few documented examples (BREEN, LUSIS and PAIGEN 1977; CHOVNIK, GELBART and MCCARRON 1977; PAIGEN *et al.*, 1975, 1976; THURIAUX *et al.* 1972; DOUGLAS and HAWTHORNE 1966, SCHWARTZ 1962). However, there is a novel type of gene cluster in eukaryotes whose significance is not yet fully understood. This is the multigene family.

As defined by HOOD, CAMPBELL and ELGIN (1975), a multigene family is a cluster of genes of common ancestry whose products have overlapping function. Examples include genes that code for histone, β-globin and antibody proteins. Although simple mechanisms have been proposed for the formation and maintenance of such gene clusters (SMITH 1976; OHTA 1976, 1977; SALSER and ISAACSON 1976), we do not yet understand why the tandem chromosomal arrangement of such functionally diverse genes has been preserved in evolution. In contrast, a class of chromosomally dispersed multigene families has recently been described in *Drosophila melanogaster* (FINNEGAN *et al.* 1977). It is reason-

able to suppose that, along with their striking chromosomal arrangement, clustered multigene families have also evolved characteristic mechanisms of regulation that may differ significantly from those associated with unique genes and dispersed multigene families (HOOD *et al.* 1975; FINNEGAN *et al.* 1977). Their mode of regulation may in part be responsible for their evolutionary persistence as blocs of genes at limited chromosomal sites. We feel that by examining the relationship between the genetic organization and the mechanism of expression of a variety of multigene systems, we may gain insight into their role as specialized units of eukaryotic chromosome function.

The silkmoth chorion genes present an appropriate multigene system for conducting such a combined genetic and biochemical study. These genes code for a set of highly specialized, small molecular weight, acidic proteins that are synthesized late in oogenesis and comprise 85% of the eggshell (chorion) of wild silkmoths of the genus Antheraea (KAFATOS *et al.* 1977) and 96% of the chorion of the commercial silkworm, *Bombyx mori* (KAWASAKI, SATO and SUZUKI 1971). The majority of the chorion polypeptides appears to be direct products of nonidentical, but homologous, structural genes. This has been demonstrated by their low levels of post-translational modification (PAUL *et al.* 1972; REGIER 1975; NADEL 1977; GOLDSMITH, RATTNER and BASEHOAR, in preparation), differences in the timing of their synthesis (PAUL *et al.* 1972; PAUL and KAFATOS 1975; REGIER 1975; NADEL 1977; KAFATOS *et al.* 1977), the small size of their messenger RNA's, which appear to be monocistronic (GELINAS and KAFATOS 1973) and the striking homologous but nonidentical amino acid sequences of purified chorion components (KAFATOS *et al.* 1977; REGIER *et al.* 1978).

The chromosomal arrangement of the chorion structural genes is presently unknown. As a first step in a detailed genetic analysis of the organization of the chorion genes, we have obtained homozygous or nearly homozygous stocks of *B. mori* that carry a wide range of electrophoretic variants of chorion proteins. We report here evidence that these electrophoretic markers behave as codominant Mendelian traits, and further, that almost all of those tested are linked to the second chromosome. This result is noteworthy because *B. mori* has a haploid set of 28 chromosomes. Further, the second chromosome carries the *Gr* (grey egg) locus, which has several alleles that produce profound pleiotropic effects on chorion ultrastructure and protein composition.

MATERIALS AND METHODS

Stocks: Stocks of silkworm races C108, Hiko and Ascoli, and strains 701 and GR were generously supplied by Y. TAZIMA and A. MURAKAMI of the National Institute of Genetics, Mishima, Japan. Stock P22 was kindly supplied by B. SAKAGUCHI and H. DOIRA of the Department of Sericulture, Faculty of Agriculture, Kyushu University, Kyushu, Japan.

Rearing and stock maintenance: Silkworms were reared generally according to the methods outlined in the *Handbook of Silkworm Rearing* (1972). Moths reared on mulberry leaves were allowed to mate spontaneously. Eggs were normally collected on Whatman #1 filter paper for 24 hr after paired matings.

Sample preparation: Eggs (untreated with formaldehyde or acid) were removed from filter paper after moistening with water to soften the natural glue. Unlaid eggs were dissected from moths into several changes of distilled water to remove haemolymph and cell debris and were

stored frozen at —20°. Eggs were cut in half and washed in distilled water according to the procedure of PAUL *et al.* (1972). Samples were solubilized using a buffer containing 6 M urea, 0.07 M dithiothreitol (Sigma), 0.05 M Tris-HCl (Schwarz/Mann Ultrapure), 0.01 M EDTA and 0.01 M lysine at pH 9.0 (EFSTRATIADIS and KAFATOS 1975). The solubilization buffer was freshly made before use with frozen aliquots of 8 M urea (Schwarz/Mann Ultrapure) which was previously decyanated by stirring at room temperature for one hr with 1/10 volume of water-washed ion exchange resin (Dowex 1×8). Samples were dissolved at an initial concentration of 10 μl per chorion for 90 to 120 min at room temperature, with occasional vortexing. Three to five chorions were routinely solubilized per sample. Carboxamidomethylation was carried out by adding ½ volume of a fresh solution containing 0.42 M iodoacetamide (recrystallized from deionized water) in 1.2 M Tris-HCl, pH 8.4, in subdued light. The reaction was terminated after 15 min by the addition of at least 3 μl mercaptoethanol (Eastman) per 100 μl total volume. These samples can be stored for up to two months at —20° without detectable alteration in the electrophoretic mobility of the proteins.

Isoelectric focusing: Isoelectric focusing was carried out according to the procedure described by EFSTRATIADIS and KAFATOS (1975), with minor modifications. Gels contained 8 M urea, 2% pH 4 to 6 ampholines (LKB), 5% acrylamide and 0.2% methylene(bis)acrylamide (Biorad). Polymerization was initiated with 0.01% TMEDA (Biorad) and 0.036% ammonium persulfate (Biorad), after degassing, and the gels were over-layered with isobutanol. Gel demensions were approximately 10 cm × 20 cm × 1 mm, requiring 30 to 35 ml of solution. Gels were stored at 5° for up to two weeks before use. Five to ten μl samples were applied to 2 mm × 18 mm strips of Whatman 3MM filter paper, which were placed 2.5 to 3 mm apart across one end of the gel, 3 to 5 mm from the edge of the basic electrode wick. The voltage was gradually increased to 800 V over a two to three hr period in 50 to 100 V increments without exceeding a maximum of 10mA, using an ISCO power supply. The gel was run at 800 V with 0 to 2° cooling for 5.5 to 6 hr, then fixed and washed briefly in ten volumes of 10% trichloroacetic acid (TCA), and subsequently washed in three changes of ten volumes of 10% TCA during the next two days with gentle rotary shaking to remove the ampholines. Gels were stained for two hrs in a solution containing 0.03% Coomassie Brilliant Blue (Sigma), 50% methanol and 10% acetic acid, then destained in 7.5% acetic acid-5% methanol with the addition of polyurethane packing sponges to absorb excess stain from the solution. Before drying gels were photographed with a yellow filter using 4 × 5 Ektapan film (Kodak), which was developed for 5 min in a 1:1 dilution of Dektol (Kodak). For permanent preservation, they were dried on Whatman 3MM filter paper on a home-made suction chamber inverted over a steam bath.

Scoring criteria: Photographs of the stained gels were scored by eye for the relative intensity, presence or absence of variant bands. The morphology and staining properties of the bands often allowed us to distinguish between intensity differences that resulted from overlap of two or more variant bands and differences that were caused by quantitative changes in a single band. Because of slight variations in the linearity of the pH gradient from run to run, we sometimes found bands resolved clearly on one gel but not on another. We report here only the data obtained for markers that could be scored consistently.

RESULTS

Electrophoretic variants and nomenclature: Races of *B. mori* have been inbred as isolated genetic lines for centuries and provide a rich source of electrophoretic variants of chorion proteins. Screening of chorion patterns produced by different races on isoelectric focusing polyacrylamide gels reveals marked qualitative and quantitative heterogeneity (Figure 1a). It should be remembered that because of the complexity of the chorion proteins, apparently quantitative differences may in fact be qualitative, resulting from lack of one or more components from a composite band (see below). On the other hand, races appear to be relatively homozygous for a given set of chorion markers. Eggs produced by different

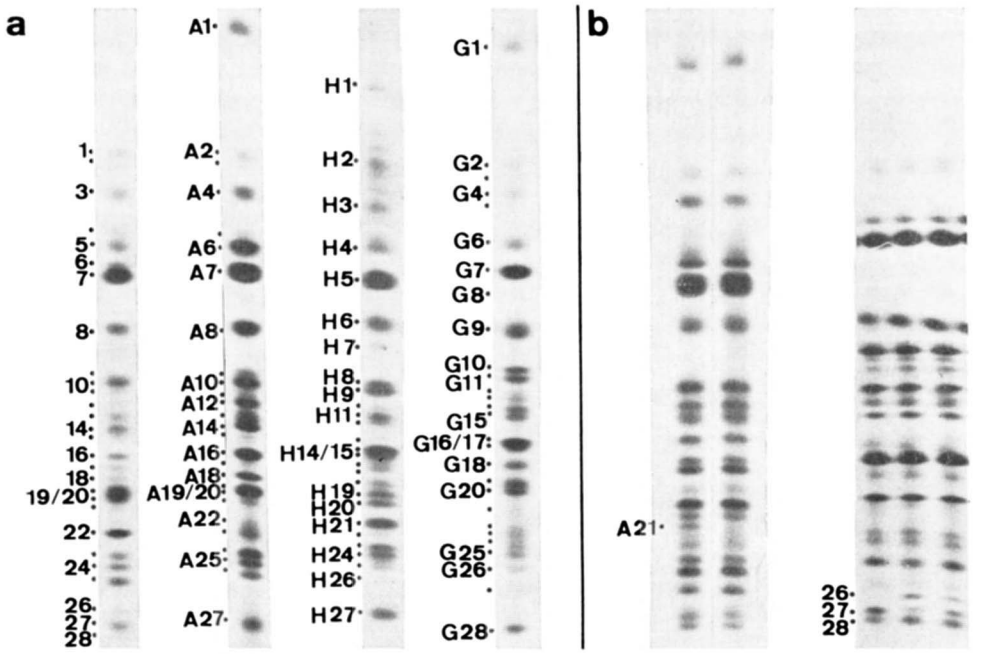


FIGURE 1.—Isoelectric focusing patterns of chorion proteins from inbred stocks of *B. mori*. Each sample was obtained from eggs laid by a single moth. Gels are oriented with the basic end on top; approximately 1/5 of the top and 1/8 of the bottom are cut off. Dots indicate major bands which are consistently resolved and are numbered consecutively from the basic to the acidic region. Alignment between samples may not be exact since samples were selected from different isoelectric focusing experiments. (a) Homogeneous patterns. C108; Ascoli; Hiko; GR. (b) Heterogeneous patterns. Variable bands are labeled. Ascoli; C108.

females from a given race usually yield identical patterns, although in some stocks we observed limited heterogeneity in a few bands (Figure 1b). True-breeding stocks derived from a few sib matings have been observed for 3 to 15 generations in our laboratory.

With the exception of C108. each stock was given a letter prefix designating the race or strain of origin: A-Ascoli; H. Hiko; G-GR, g-701. All bands that were consistently resolved on pH 4 to 6 isofocusing gels were numbered consecutively in a given stock from the basic to the acidic region. Thus, bands with identical pKi originating in different stocks could be assigned different numbers. We felt that this system of nomenclature would in part avoid the assumption that bands with the same electrophoretic mobility represented identical gene products. Hybrid bands marked with an asterisk(*) in some gels did not co-migrate with known parental standards and thus could not be identified unequivocally. These were numbered according to our best estimate, based on their mobility and intensity relative to neighboring bands resolved on the same gel. In cases of overlap between two parental markers which precluded identification in the hybrids, we designated the progeny band with both parental markers separated by a slash (*e.g.*, 21/A21).

Heterozygosis: Protein patterns from 10 to 25 egg batches obtained from a single race showed minimal heterogeneity, suggesting that stocks were nearly homozygous. However, the possibility remained that some markers existed as balanced heterozygotes and would remain undetectable until outbred with appropriate stocks.

On the other hand, analysis of the F_1 progeny gives a powerful test for heterozygosity if the structural genes behave in a co-dominant fashion. Crosses made between parents homozygous for their respective chorion structural genes should yield a single F_1 chorion protein pattern consisting of the observable electrophoretic variants from both parents in approximately half the parental amounts. If one parent is homozygous and the second heterozygous for a given structural gene, the progeny will segregate into two phenotypic classes, each producing one of the variant proteins originating in the heterozygote. Clearly, a greater number of protein patterns may appear in the F_1 with increased heterozygosis. The number of F_1 phenotypes would also be increased by the existence of more than one chorion linkage group.

We limited our initial studies to those crosses yielding no more than two classes of F_1 progeny in order to minimize the complications introduced by heterozygosis. This restricted the maximum number of alleles that could be identified at a given locus to three. The production of a single F_1 class suggested that the parents were indeed homozygous for their respective sets of variants. This observation could in part be confirmed by examination of parental chorion patterns over several generation, as well as by subsequent testcrosses.

Among nine different pair matings, which included a total of eight stocks, only three produced more than two chorion patterns in the F_1, two of them involving the stock designated Sunrei. Two parental combinations yielded two classes of F_1 progeny, indicating that one of the parents was probably heterozygous (see below). Stock P22 showed the most variability observed within a single line and was not used in these experiments; five proteins in the most acidic pH range varied in both intensity and presence. Thus, at our level of resolution, many inbred silkworm stocks are homozygous for chorion genes.

Genetic rationale and synopsis of the results: The purpose of this study was to determine whether the identifiable electrophoretic variants in the test stocks belong to the same or different linkage groups. We made an F_1 by crossing two stocks that produced dissimilar protein patterns to determine the dominance relationships of the variants. We then carried out reciprocal testcrosses to each of the parents to determine whether or not there was linkage. Linked markers should cosegregate in the testcross progeny, producing chorion protein patterns identical to either the homozygous parent or the F_1. Unlinked markers should assort independently and generate new electrophoretic patterns that resemble neither the parental nor the F_1 phenotype. The number and variety of recombinant patterns should vary with the number of linkage groups (Figure 2).

For the testcross, we mated an F_1 female with one of the parental males. Since there is no detectable crossing over in *B. mori* females, this should eliminate the effects of intrachromosomal recombination. The latter would add considerably to the complexity of the system if a large number of linkage groups were involved.

Progeny from each pair mating were analyzed separately. Thus, where duplicate crosses were carried out, we report the data obtained from each set of parents (pair matings) separately.

The complexity of individual chorion patterns and the lack of a biochemical or functional test for specific chorion gene products make identification of alleles unfeasible in this study. Thus, we used a single test race, C108, as the reference standard for each set of crosses. In this manner, we were able to accumulate linkage data by comparing the inheritance of markers found in each stock relative to C108.

Table 1 presents a list of all chorion markers used in this study. Shown in boldface are those components that served as qualitative markers, plain type those that showed apparently quantitative differences, and in parenthesis those that showed complex inheritance patterns, which we interpret as due to heterozygosity (see below). The results can be summarized by stating that, with but one exception, in each type of testcross the entire set of markers derived from each parent co-segregated. Because of overlaps between the three sets of C108 markers analyzed in each cross, (*e.g.*, bands 6, 10 and 25), it follows that all the C108 markers listed in the first part of the table are linked. This includes nine qualitative markers and six apparently quantitative ones, yielding a total number of 15 linked C108 markers. In addition, four markers tested in GR are linked, as are

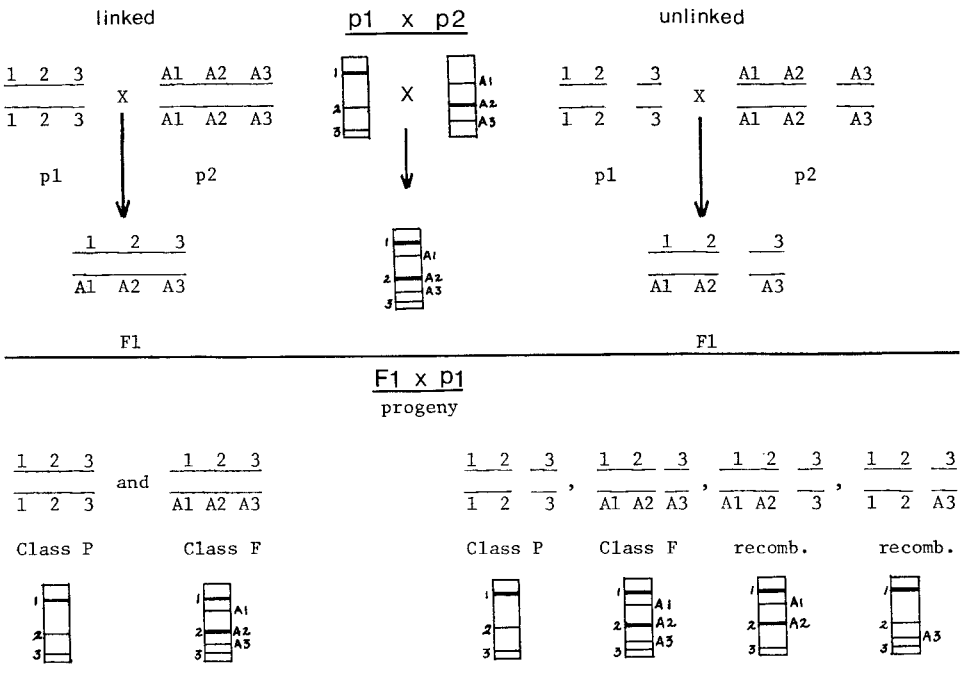


FIGURE 2.—Mating scheme to test for chorion gene linkage. Each number corresponds to a structural gene whose product yields a protein at the indicated gel position; chromosome map positions are arbitrary. P_1 and P_2 are different homozygous inbred stocks. Depicted are theoretical chorion protein patterns produced by parental, F_1, and testcross individuals. Left, three linked genes. Right, two linked genes and one unlinked gene.

TABLE 1

Chorion markers tested for linkage in crosses between C108 and other stocks

Stocks crossed	C108 markers	Other stock markers
a. Linked markers		
C108 and Ascoli	**6**, 10, **11**, **15**, ***17**, 19/20, (21),**22**, 25, **26**, **28**	**A1**, A6, A7, A10, A12, **A15**, A16, A18, (A21), **A22**, A23, (A24), (A26)
C108 and Hiko	**6**, *9, 10, **11**, **14**, **15**, 17, 25	**H1**, H5, *H8, H14/15, *H22
C108 and GR	**6**, 10, **11**, 14, 15, **17**, **21**, 22, 23, 24, 25, (26)	**G1**, **G10**, **G17**, **G18**
b. Unlinked markers		
C108 and Ascoli	**3**	

Markers listed in boldface are qualitative and in plain type are quantitative or morphological variants. Markers listed in parentheses show complex segregation patterns interpretable as due to heterozygosity in the parental strains.

five markers in Hiko and 13 markers in Ascoli, if we include the markers that show complex segregation patterns (see below).

In the testcrosses involving C108 and GR, the GR-derived chorion protein markers invariably co-segregated with two morphological markers known to be carried on chromosome *2*, namely *Gr* (grey egg) and *Y* (yellow cocoon). These results establish that the chorion protein markers are linked to the second chromosome.

Parental stocks: The parental stocks used in these studies were races C108, Ascoli and Hiko, and a stock of unknown racial origin designated GR (Figure 1a). The protein patterns observed in both the Hiko and GR stocks were invariant during the course of this study, four and eight generations, respectively. In contrast, a few individual inbred females from the other two stocks produced more than one phenotypic class (Figure 1b). Race C108 displayed variability in bands 26, 27, and 28, which showed an approximate two-fold range in intensity, and sometimes lacked one or both of bands 26 and 28. The Ascoli stock gave rise to protein patterns differing by the presence or absence of a single band (A21). A likely explanation for these variations is the presence of a different allelic form of the genes that was undetected in the isofocusing patterns lacking the band because of co-migration with other proteins. However, we cannot rule out the possibility that the genes in question were deleted, or not expressed.

F_1 Generation: Pair matings were carried out using C108 × Ascoli, C108 × Hiko, and C108 × GR. Except where noted, the results reported here were obtained from the offspring of a single pair mating. The progeny displayed chorion patterns consistent with the behavior of individual bands as the products of normal Mendelizing genes exhibiting co-dominance. Thus, the F_1 produced all of the major parental bands resolvable on the isofocusing gels, at staining intensities ranging from approximately one-half to equivalent to parental levels (with the exception of heterozygous markers; see below) (Figure 3). This indicated no unusual gene dosage effects or interaction between gene products of different chorion loci for the majority of bands observed. In particular, there did

not appear to be modifiers specific to any of the stocks that were capable of selectively altering the electrophoretic mobility of the resolved chorion bands.

The offspring derived from one cross of C108 with GR and one with Hiko, respectively, were consistent with the idea that the parents were homozygous (Figures 3b and c). The C108/Hiko progeny failed to express one parental marker (band H8). However, this band may have fused with adjacent C108 markers (band 9/10) in the samples examined (Figure 3b). On the other hand, we observed two phenotypic classes in the progeny derived from a cross of C108 with Ascoli and a second C108 cross with Hiko. The two C108/Ascoli classes were characterized by either the presence (five cases) or absence (three cases) of bands 26 and 28 (Figure 3a). Again this was consistent with our earlier observations on the variability of these markers in the parental C108 stock. The two C108/Hiko classes were also characterized by the variable expression of two sets of chorion markers. One class (three cases) was identical to the homozygous F_1 cross, again lacking band H8 (Figure 3b). The second class (five cases) expressed band H8 as well as two other bands at higher intensity (bands 23/H23 and 24a/H25); further, it lacked two markers expected from the combined parental phenotypes (bands 19 and 25/H26). None of these bands had been observed to be variable in the parental stocks; however, this may have resulted from our failure to resolve them under the usual conditions on the isofocusing gels in that pH zone.

Testcrosses: We selected individual F_1 females at random to carry out test-

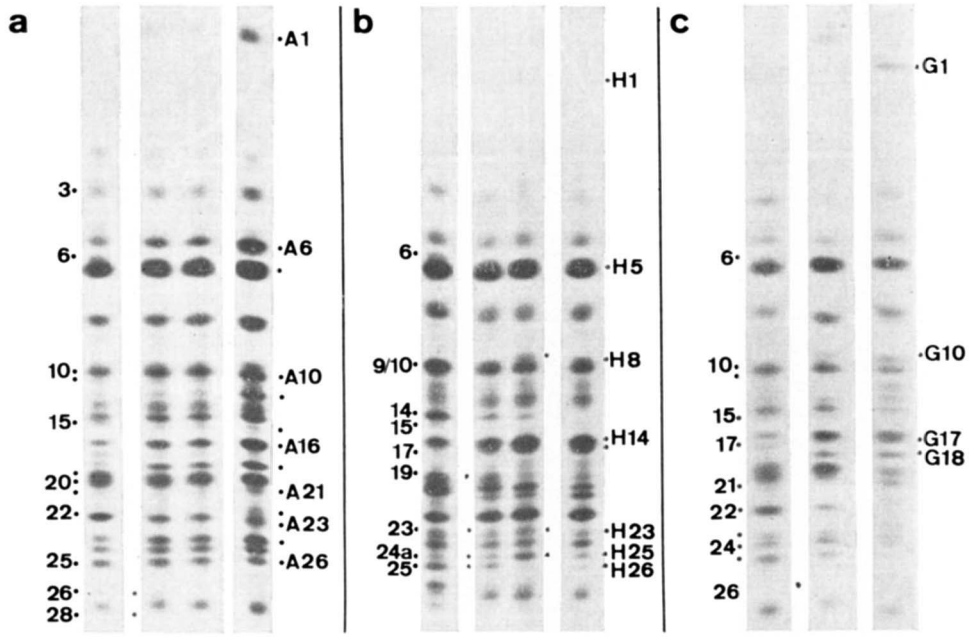


FIGURE 3.—Isoelectric focusing patterns of chorion proteins from F_1 progeny. Indicated are markers mapped in subsequent testcrosses, as discussed in the text. (a) C108; C108/Ascoli; C108/Ascoli; Ascoli. (b) C108; C108/Hiko (crosses 1 and 2); C108/Hiko (cross 2); Hiko. (c) C108; C108/GR; GR.

crosses to purebred males of either parental type in pair matings. In every case we observed two alternative sets of markers that were expressed in an all-or-none pattern in the testcross progeny, providing strong evidence for linkage. Thus from each type of testcross, we recovered two major phenotypic classes, which we designated Class F and Class P. Class F produced a protein pattern displaying all the electrophoretic variants noted in the F_1 generation and was usually indistinguishable from it. Class P produced mainly variant bands characteristic of the testcross male. The inheritance patterns of a few exceptional markers were consistent with the hypothesis that they belonged to the same linkage group, but were heterozygous in the parental stocks. Thus, they were found in association with Class F or Class P progeny, as expected from their parental origin on the hypothesis of linkage, and did not appear to yield patterns resulting from random assortment. Further, most of these variable markers had been observed to be heterozygous in the parental and F_1 protein patterns.

The three sets of C108 markers that could be scored in the crosses involving Ascoli, Hiko and GR included 16 C108 isoelectric focusing bands out of a total of 28 proteins resolved. As noted above, each of the three subsets behaved as a linked group including 15 of the 16 markers tested in C108 (Table 1). Our conclusion is reinforced by the fact that 13 of these markers were observed to co-segregate in more than one testcross.

Testcrosses involving C108 and Ascoli: The most striking phenotypic results were obtained from the testcrosses involving races C108 and Ascoli. In reciprocal crosses we observed a total of 23 variant bands distributed among Class F and Class P segregants, which were of approximately equal frequency (Table 2). Eleven of these markers originated in the Ascoli race and 12 in C108. Class F of the testcross with C108 was characterized by eight Ascoli-derived bands (A1, A6, A7, A12, A16, A18, A22 and A23) and was essentially indistinguishable from the F_1 (Figure 4a). Class P was marked by three C108-derived bands (6, 26 and 28) and produced a protein pattern indistinguishable from C108. As

TABLE 2

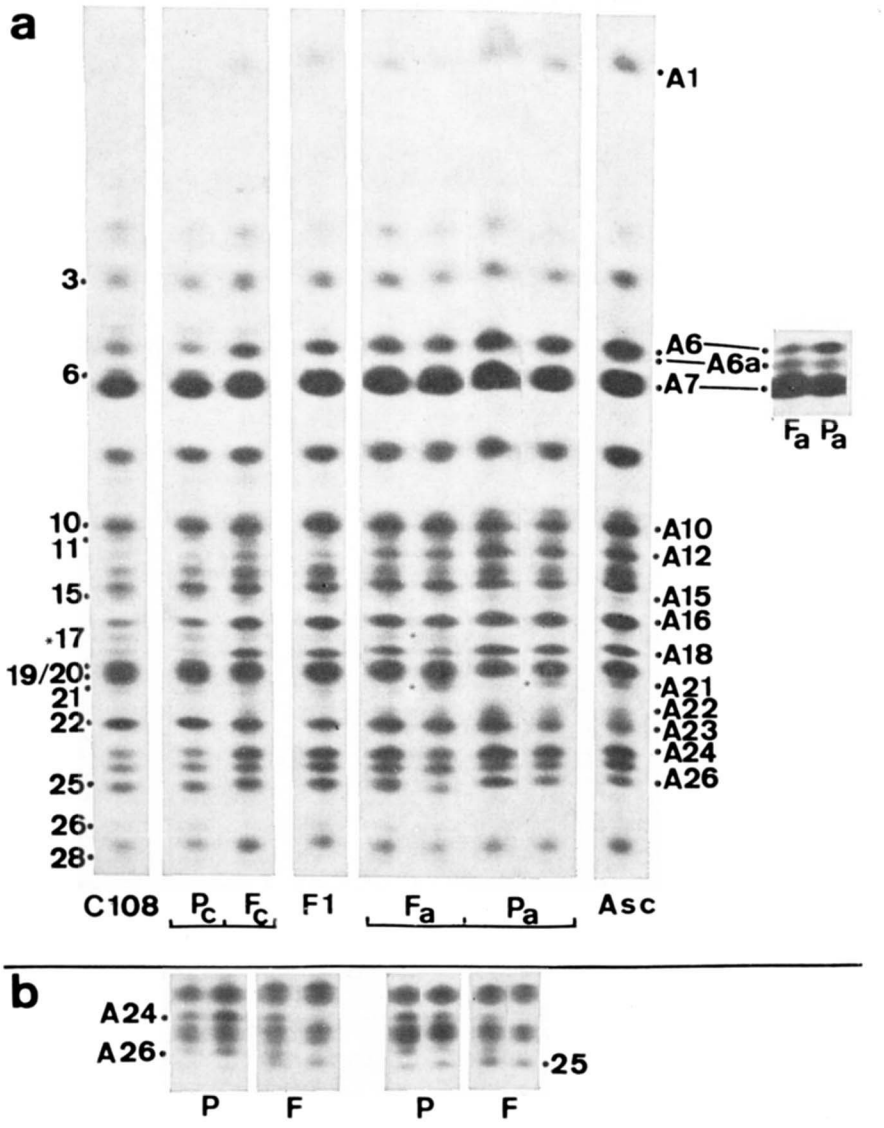
Segregation of linked chorion markers in C108 vs. *Ascoli testcrosses*

Cross	Markers	No.	Markers	No.	Total	Ratio *F*:*P*	*p*
C108/Ascoli × C108	**A1**, A6, A7, A12, [A16], [A18], **A22**, [A23]	14	**6, 26, 28**	18	32	1.28	$0.50>p>0.30$
C108/Ascoli × Ascoli							
Cross 1	[10], **11**, **15**, ***17**, 19/20, [22], 25, **26**, **28**	6	A1, A6, [A10], **A15**, A16, A18	6	12	1.0	$p>0.98$
Cross 2	[10], **11**, [15], 19/20, [**22**], 25	9	A1, A6, [A10], A12, **A15**, A18	9	18	1.0	$p>0.98$

Each cross represents data obtained from a single pair mating. All bands listed in one class are either missing or found at lower intensity in the other class. Markers listed in boldface are qualitative, in plain type are quantitative, and in brackets are distinguishable by morphology. P = probability associated with χ^2 test of F:P ratio of 1.0.

expected from the parental and F_1 patterns, only bands A1, A22, A23, 6, 26 and 28 were qualitatively unique to a given class; the remainder were observed as variants in staining intensity or band morphology (*e.g.*, fuzzy *vs.* sharp, as in bands A23 and 22, Figure 4a).

The absence of bands 26 and 28 was an unexpected feature of the Class F segregants, since they had been present in the F_1. However, this finding is consistent with our previous observations on the variability of these bands in C108 and F_1 individuals. We conclude that the band 26 and 28 chorion genes are linked to the other C108 markers, but were not present in the testcross male. Thus, they were expressed only in the Class P segregants, which inherited the C108 homologue carrying the band 26 and 28 alleles from the F_1 parent.



These results were confirmed in two separate testcrosses with Ascoli. In both we again observed two distinctive phenotypic classes of equal frequency (Table 2, Figure 4a). The Class F progeny from both crosses resembled the F_1 phenotype overall and displayed a set of six bands characteristic of C108 (bands 10, 11, 15, 19/20, 22 and 25), of which two appeared as variants in intensity (bands 19/20 and 25) and the remainder were distinguished by morphology and gel position (*e.g.*, band 15 more basic than band A15, Fig. 4a). Class P segregants displayed four Ascoli markers at higher intensity than the Class F segregants (bands A1, A6, A10, and A18) and one of distinctive gel position (band A15). Two other markers appeared in patterns consistent with the possibility that they were linked to the other chorion genes, but were resolvable in only one of the two sets of samples (bands A12 and A16).

Six markers behaved as if they were heterozygous in the parental stocks, displaying different behavior in cross one and cross two (bands *17, 26, 28, A21, A24, and A26). We have already demonstrated the variability of bands 26 and 28; evidently, the alleles coding for these proteins were present only in the cross one F_1 female, since they failed to appear in the Class F samples of cross two (Figure 4a; Table 2). Unfortunately, we were unable to verify this conclusion by direct examination of the parental egg batches, which were not recorded in the course of our study. Band *17 clearly originated in the C108 stock, as it was present in the C108 but not in the Ascoli standard included in the gels used to resolve cross one samples. However, the resolution did not allow us to verify its identity with band 17. Our failure to note a band migrating in this region as a heterozygous marker in the C108/Ascoli F_1 samples was probably due to its overlap with the much more intensely staining band A18 (Figure 3a).

The behavior of bands A24 and A26 suggested that their structural genes were linked to the other Ascoli markers in the F_1 female, but were present on only one chromosome of the testcross male. Linkage was indicated by the fact that the two bands were expressed in parallel; they were either both absent, both present at low intensity, or both at high intensity (Figure 4b; Table 3). The zero, low and high intensity phenotypes appeared in an approximate 1:2:1 ratio, suggestive of a heterozygous cross in which intensity variations are the result of varying gene dosage. A key diagnostic feature was that the null phenotype was limited to the Class F segregants. These results can be explained by the hypothesis that Class F is defined by a single chromosome bearing only the C108 markers, which is inherited from the F_1 female (Figure 5). If so, Class F would fail to express the bands if the homologue contributed by the heterozygous testcross male lacked the

FIGURE 4.—Isoelectric focusing patterns of chorion proteins from progeny of C108/Ascoli testcrosses. Numbered bands were scored as discussed in the text (Table 2). (a) Complete isoelectric focusing patterns. C108; testcrosses to C108: P_c-Class P, F_c-Class F; F_1; testcrosses to Ascoli: F_a-Class F, P_a-Class P; Ascoli. Starred bands (*) tentatively identified as 17 (upper) and 21/A21 (lower). Inset, resolution of band A6 in separate gel run. Band at position A6a is observed in both C108 and Ascoli; band at position A6 is not observed in C108. (b) Acidic portion of isoelectric focusing patterns from testcrosses to Ascoli, resolved in separate gel run, illustrating variations in bands A24, A26 and 25 (Table 3). Left, Class P and Class F segregants, cross 1. Right, Class P and Class F segregants, cross 2.

TABLE 3

Segregation of heterozygous or unlinked chorion markers in C108/Ascoli × Ascoli testcrosses

Cross	Phenotype	No. progeny Class F	Class P	Total
a. Markers heterozygous in testcross male				
1	A21 null, A24 high intensity, A26 high intensity	0	2	2
	A21 sharp, A25 low intensity, A26 low intensity	2	4	6
	A21 diffuse, A24 null, A26 null	4	0	4
	Total	6	6	12
2	A21 low intensity, sharp; A24, A26 high intensity	0	4	4
	*21 high intensity, diffuse; A24, A26 low intensity	4	5	9
	A24, A26 null	5†	0	5
	Total	9	9	18
b. Unlinked markers				
1	3 high intensity	5	4	9
	3 low intensity	1	2	3
	Total	6	6	12
2	3 high intensity	9	4‡	13
	3 low intensity	0	5	5
	Total	9	9	18

† Bands *21 and A21 could not be scored in Class F samples because of overlap with bands 19/20.

‡ Two of these samples have intensity intermediate between "high" and "low" intensity classes.

A24 and A26 genes, and would express them at low intensity if the Ascoli-derived chromosome carried the A24 and A26 genes. Further, since Class P displayed high and low intensity phenotypes but no null, and according to our hypothesis is defined by the Ascoli marker chromosome inherited from the F_1 female, the latter must carry the genes for bands A24 and A26.

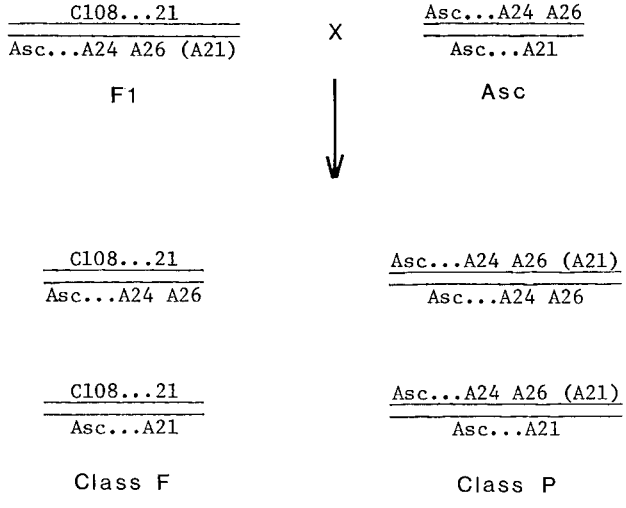


FIGURE 5.—Mating scheme with presumed genetic constitution of parents for markers 21, A21, A24, and A26 in C108/Ascoli × Ascoli testcrosses. Chromosome map positions are arbitrary. (A21) is present only in cross 2. C108 and Asc refer to the other linked chorion markers.

Band(s) migrating at position 21/A21 showed striking patterns of expression relative to bands A24 and A26, which suggested that the A21 gene, by similar arguments, was heterozygous in our crosses (also demonstrated in the parental chorion patterns; Figure 1b), and allowed us to reconstruct a likely parental chromosomal constitution with respect to these three markers. In cross one, band 21/A21 appeared only in the Class P samples, which displayed bands A24 and A26 at low intensity. In cross two, we found a reciprocal pattern of expression with respect to bands A24 and A26; high intensity samples of the latter displayed band 21/A21 at low intensity, and *vice versa* (Figure 4b; Table 3). Thus, in both crosses there appeared to be a copy of the A21 gene linked to the testcross chromosome that did not carry the A24 and A26 alleles; in cross two, there also appeared to be a second copy on the F_1-derived Ascoli marker chromosome (Figure 5). In addition, both F_1 females evidently carried gene 21 on the C108 marker chromosome, since no Class P samples were null for a band at that gel position. This prediction could not be confirmed by observation of the Class F samples in cross two, in which band 19/20 masked the critical region of the gel (Figure 4a).

The gene for band 3 behaved as if it were not linked to either the C108 or Ascoli markers. The band was present in samples at high and low intensities in ratios of 9:3 and 13:5 in crosses one and two, respectively (Table 3). All but one of the Class F progeny carried the band at high intensity, while the two phenotypes were distributed at random among the Class P samples. The presence of the low intensity band 3 sample in Class F is difficult to reconcile with association of the band 3 gene with the C108 linkage group. Further, we could find no correlation between the Class P phenotypes for band 3 and the segregants defined by bands A21, A24 and A26.

Testcrosses involving C108 and Hiko: A similar set of testcrosses was performed using races C108 and Hiko. The inheritance patterns confirmed the previous results, with two classes of offspring appearing in roughly equal numbers (Figure 6; Table 4). Class F of the C108 testcross was characterized by three markers originating in Hiko (bands H1, H5 and H14/15), of which one appeared exclusively in this class (band H1) Class P displayed seven markers from C108 (bands 6, 10, 11, 14, 15, 17 and 25), of which four were unique (bands 6, 11, 14 and 15). In the reciprocal Hiko testcross we again observed two segregant classes of equal frequency (Figure 6; Table 4). Class F displayed two C108 markers at high intensity (bands 14 and 25) and two other bands which could not be unequivocally identified with known markers (band *9/H8 and *H22) because of differences in relative pKi and intensity. Class P was primarily characterized by the absence or low intensity of these markers.

Several bands expected from the Hiko phenotype in the pH range between bands H14/15 and H19 did not focus in the testcross samples. This appeared to be the result of peculiarities of the isoelectric focusing conditions and not necessarily indicative of their absence from the solubilized eggs, since proteins in this region often remain unresolved in samples in which they are known to be present. Although the samples were run on at least five separate gels, we

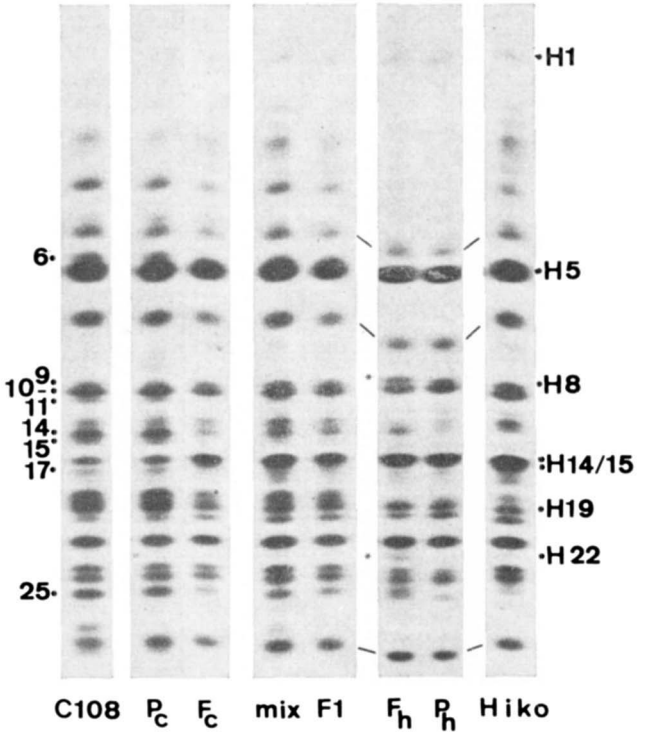


FIGURE 6.—Isoelectric focusing patterns of chorion proteins from progeny of C108/Hiko testcrosses. Numbered bands were scored as discussed in text (Table 4). C108; testcrosses to C108: P_c-Class P, F_c-Class F; MIX — 1:1 mixture of C108 and Hiko samples; F_1; testcrosses to Hiko: F_h-Class F, P_h-Class P; Hiko.

never observed distinct focusing of the bands in question either in testcrosses or controls.

Linkage to the second chromosome: Chromosome *2* bears a number of sites that play a significant role in the expression of chorion phenotype. *Gr*, mapping at 6.9 (TAKASAKI 1962), is a prominent locus affecting chorion morphology. The

TABLE 4

Segregation of linked chorion markers in C108 vs. *Hiko testcrosses*

Cross	Progeny class F Markers	No.	Progeny class P Markers	No.	Total	Ratio F:P	p
C108/Hiko × C108	**H1**, H5, H14/15	22	**6**, (10), **11**, **14**, **15**, (17), 25	15	38	1.47	$0.30>p>0.20$
C108/Hiko × Hiko	*9/H8, 14, *H22, 25	16	Class F bands missing or low intensity	16	32	1.0	$p>0.98$

Each cross represents data from a single pair mating. All bands listed in one class are either missing or found at lower intensity in the other class. Markers listed in boldface are qualitative, in plain type, quantitative and in parentheses, distinguishable by morphology. P = probability associated with χ^2 test of $F:P$ ratio of 1.0.

TABLE 5

Segregation of linked chorion markers in C108 vs *GR testcross*

Cross	Progeny class F Markers	No.	Progeny class P Markers	No.	Total	Ratio F:P
GR/C108 × C108	**G1, G10, G17, G18,** Gr, Y	8	**6,** (10), **11,** 14, 15, **17, 21,** 22, 23, 24, 25, $_{+}$Gr, $_{+}$Y	16	24	0.50

Data was obtained from a single pair mating. All bands listed in one class are either missing or found at lower intensity in the other class. Markers listed in boldface are qualitative, in plain type are quantitative, and in parentheses are distinguishable by morphology.

four known spontaneous *Gr* alleles, *Gr*, Gr^{16}, Gr^{B}, and Gr^{col}, give rise to opaque, milky chorions or thin, collapsing eggshells, which are readily distinguished by eye from the translucent wild-type chorion. Gr^{16} is dominant, Gr^{col} recessive, and *Gr* and Gr^{B} show incomplete dominance, with distinctive morphologies in homozygous and heterozygous condition. All allelic combinations that produce a phenotypic change in chorion structure are accompanied by reduced amounts of the number and kinds of chorion bands expected from analysis of the corresponding wild-type protein patterns (KAFATOS *et al.* 1976; NADEL 1977; unpublished observations). Their mechanism of action is not fully understood, but is presently under investigation.

In addition to the *Gr* locus, X-ray-induced chromosome breaks occurring in the zone between the *S* locus (Striped larva) at map position 6.1 (CHIKUSHI 1972) and locus 6.9 can result in the induction of grey phenotypes. The radiation-induced *S* mutation itself acts as a suppressor of some of the *Gr* alleles (TAKASAKI 1962; in TAZIMA 1964). The suppressor function has not been mapped directly and may not correspond to the *S* locus.

To determine whether the chorion markers observed in our crosses were linked to chromosome *2*, we crossed C108 to GR, a homozygous *Gr*-bearing stock of unknown racial origin that also carries the chromosome *2* marker *Y* (yellow hemolymph and cocoon) at locus 25.6 (TAZIMA 1964). C108 bears the recessive allele for *Y* (white cocoon). As expected, all the F_1 females spun yellow cocoons and laid phenotypically grey (*Gr*/+) eggs; the F_1 chorion pattern displayed variant bands characteristic of both parental stocks (Figure 3c). In the subsequent testcross to C108, we observed segregation of egg batches into the grey

TABLE 6

Segregation of heterozygous chorion markers in GR/C108 × C108 testcross

Phenotype		No. progeny Class F	Class P	Total
26 high intensity		0	2	2
26 low intensity		5	14	19
26 null		3	0	3
	Total	8	16	24

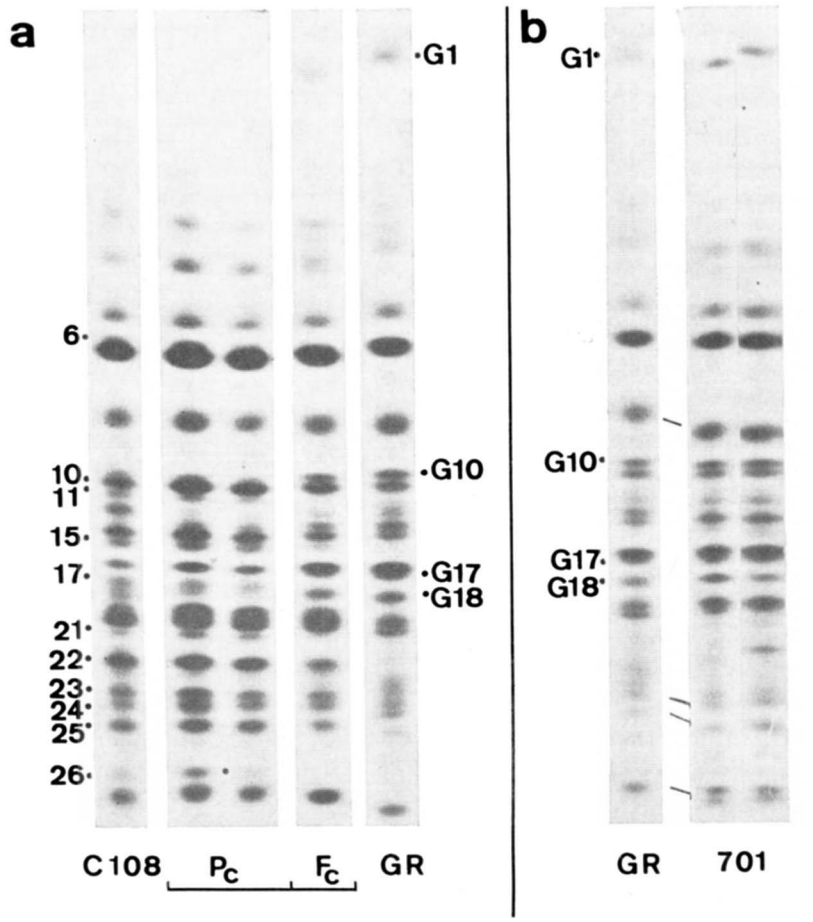


FIGURE 7.—Isoelectric focusing patterns of chorion proteins from progeny of C108/GR testcross and stocks carrying the *Gr* gene. Numbered bands were scored as discussed in the text (Table 5). (a) Testcross to C108. C108; P_c-Class P; F_c-Class F; *Gr*. (b) Stocks GR and 701. GR-*Gr*/*Gr*; 701-*Gr*/*Gr*; 701-+/+.

heterozygote and wild-type chorion phenotypes, with corresponding linkage of the *Gr* marker to *Y* as expected (Table 5a). Class F and Class P isofocusing patterns showed complete correlation with the two types of segregants. In all we observed eight grey Class F egg batches and 17 wild-type Class P batches. The low yield of grey egg batches may be the result of reduced hatchability and viability of the *Gr*-bearing larvae, which has also been observed in other laboratories (TAKASAKI, 1962).

The Class F isofocusing pattern appeared to be identical to the F_1, and was distinguished by four electrophoretic variants characteristic of the GR stock (bands G1, G10, G17, and G18), all of which were qualitatively unique (Figure 7a). Class P chorions appeared identical to C108 and were marked by ten variant bands (bands 6, 10, 11, 15, 17, 21, 22, 23, 24, and 25), of which four were unique (bands 6, 11, 17, and 21). Band 26 introduced by C108 again behaved as if it

were heterozygous in the C108 testcross male and present in the F_1 female. It appeared in five of the eight Class F samples and in all of the Class P samples at relative intensities that correlated with this hypothesis (Table 6).

The GR bands followed in this study did not appear to be produced by the action of the *Gr* mutation itself. Proteins with identical electrophoretic mobility are observed in wild-type chorions produced by 701, another stock that carries the *Gr* mutation (Figure 7b). The homozygous *Gr* chorion patterns in both GR and 701 are nearly identical (Figure 7b).

DISCUSSION

Our experiments demonstrate by formal genetic analysis that a large set of *B. mori* chorion structural genes are linked. It can be argued that the presence of a single modifier gene in one stock that alters the electrophoretic mobility of a set of chorion proteins, or produces a set of modified proteins from a single precursor, could be mistaken for a set of linked genes in this kind of study. This seems unlikely on the basis of both genetic and structural data. It is difficult to reconcile the behavior of a single modifier gene with the appearance of the F_1 co-dominant chorion patterns we observe, unless it acted on a single site on each modifiable polypeptide chain, and was dose limited. The presence of two different forms of a modifier in the F_1 (inherited from each parent) should result in competition between them in the F_1 cytoplasm. If there is more than one modification site on each protein chain, competition at each site should generate a variety of products not necessarily corresponding in pKi with parental forms of the mature chorion polypeptides. On the other hand, competition for modification of a single site would produce only parental molecular forms, but in proportion to the relative efficiencies of the modifier functions. This would give the observed F_1 chorion pattern, with roughly equal levels of each parental protein band, only if the modification processes were equally successful in competing. This possibility, however unlikely, is not ruled out by our experiments.

Direct evidence that electrophoretic mobility differences in chorion proteins correspond to differences in primary structure has been obtained by REGIER *et al.* (1978) for the silkmoth, *A. polyphemus*. Equivalent data is not available in *B. mori*. However, we conducted a survey of inbred stocks, including all those reported here, for evidence of post-translational modification by comparing the isoelectric focusing patterns of newly synthesized and aged chorion proteins. We were unable to detect any precursor or product bands, although we observed a small fraction of labile components in early chorionating follicles (GOLDSMITH, RATTNER and BASEHOAR, in preparation). This result is in essential agreement with findings in *A. polyphemus*, in which approximately 30% of incorporated leucine exhibits altered charge as a result of aging in pulse/chase experiments (REGIER 1975). Further, only trace amounts of amino sugars, a likely modifying residue, are found in the soluble chorion proteins of *B. mori* and a variety of Saturniids (KAWASAKI, SATO and SUZUKI 1971).

Our results might also be explained by the possibility that all *B. mori* strains carry the identical complement of chorion genes, but only a subset are expressed

in a given race as the result of the differential action of a series of regulatory genes, or the synthesis of defective untranslated mRNA's (KAN *et al.* 1975). These possibilities could be tested by demonstrating the presence of "silent" structural genes or defective mRNA's using molecular hybridization as a tool (KAN *et al.* 1975; TOLSTOSHEV *et al.* 1976). Experiments of this kind are being carried out in this and other laboratories (F. C. KAFATOS, T. MANIATIS, personal communication).

A major assumption of this study is that most of the between-race intensity variations observed in the isofocusing patterns are not quantitative, but are caused by co-migration of qualitatively different proteins. Individual band staining properties and morphology are often distinctive, and in many cases allowed discrimination between overlapping bands derived from different strains. In some cases we have also been able to distinguish co-segregating bands by differences in synthetic periods. Two dimensional gel electrophoresis (O'FARRELL 1975) of chorion samples in which focused bands are rerun in the presence of SDS resolves up to 60 spots, indicating considerable overlap in components separated by isofocusing alone. A preliminary two-dimensional analysis of Class F and Class P chorions produced in the C108/Ascoli × C108 testcross reveals complete identity of these samples with the F_1 and parental patterns, as expected (GOLDSMITH, BALIKOV, RATTNER and BOCK, unpublished observations). While this method is not feasible for large-scale screening, the improved resolution warrants its use in a reexamination of representative testcross samples.

The finding of only a single unlinked chorion marker thus far opens up the question of the evolutionary basis for the maintenance of linkage among such functionally overlapping genes. All the markers observed in this study were chosen without regard for their biochemical properties or role in chorion ultrastructure. *B. mori* chorion components fall into four molecular weight classes, designated A (average molecular weight 9,000 daltons), B (13,000 daltons), C (18,000 daltons) and Hc (18,800 daltons), which appear to be encoded by evolutionarily divergent but related families of genes (KAFATOS *et al.* 1977; REGIER *et al* 1978). It is crucial for our understanding of the structure of multigene families to determine whether members of all four chorion subgroups are linked, and further, whether the linked genes are clustered. The chorion subclass can be established for a set of proteins using the two-dimensional analysis described above. It is almost certain that the majority of markers tested so far will belong to the B proteins, which comprise perhaps 60% of the total chorion mass. Further, it is clear that we are not following the behavior of the Hc proteins, which stain poorly with Coomassie blue and are minor components interspersed throughout the isofocusing range tested (REGIER 1975). Whether chorion genes are tightly linked can be determined with a testcross under conditions in which crossing over can be observed, using a homozygous parental female and F_1 male, and is presently under investigation.

The linkage of chorion genes to chromosome *2*, which carries the *Gr* locus, is a potentially exciting feature of this system. The *Gr* mutation has a pleiotrophic effect on chorion production, and in some respects behaves like a regulatory site. Gr^B causes a severe reduction in the number of chorion components synthesized,

and also displays a reduction in the spectrum of mRNA's (NADEL 1977). Gr^{col} acts posttranslationally, resulting in the secondary loss of newly formed components (NADEL 1977). It is difficult to reconcile these two phenotypes with the action of a single regulatory gene. Determining the chromosomal organization of the chorion structural genes relative to the *Gr* locus would be an important step in elucidating its role in chorion biosynthesis. It would be of particular interest to determine whether the *Gr* genes act in *cis* or *trans*, and whether the Gr^{B} mutation is a deletion of part of a chorion gene cluster, or a regulatory site. Experiments are now under way to answer these questions.

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