EXPRESSION OF GENES AT THE AGOUTI LOCUS AND MITOTIC ACTIVITY OF THE HAIR BULB OF THE MOUSE¹

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THE action of genes at the agouti locus, in combination with "wild" genes at other color loci, results in coat color phenotypes which range from all black (a/a) to all yellow ($A^y/-$). Between these two extremes is the agouti phenotype ($A/-$) which is typically characterized by the presence of a subapical yellow band on an otherwise black hair. These observations indicate that alleles at the agouti locus control the nature of the pigment manufactured by melanocytes in the hair bulb, i.e., whether it is black (eumelanin) or yellow (phaeomelanin), or whether both eumelanin and phaeomelanin will be produced (the agouti pattern). The action of the agouti gene (A) apparently is to control a mechanism which causes a rapid and rhythmic switch between the production of black and yellow pigment during the course of hair growth. The expression of this activity however is conditioned by the environment so that the agouti pigment pattern varies according to the integumentary environment of the hair follicle, the hair growth generation, and the hair type (GALBRAITH 1964). Moreover, genotypically agouti melanocytes do not produce yellow pigment if they reside in hair bulbs that exceed 110μ in diameter (GALBRAITH 1969), nor do they produce yellow pigment when in extrafollicular environments (SILVERS 1957; MARKERT and SILVERS 1956).

These observations indicate that the hair follicle itself plays an important role in the expression of alleles at the agouti locus. This view is supported by the work of SILVERS and RUSSELL (1955) and SILVERS (1958a, b, 1965) who have demonstrated that the melanogenic response of pigment cells is in accordance with the agouti-locus genotype of the follicle in which they reside rather than their own agouti-locus genotype. Thus the inherent capacity of the melanocyte is to produce black pigment, and phaeomelanin is produced only in the local environment of a hair follicle of appropriate agouti-locus genotype.

The nature of this environment is not known, but it has been suggested that the production of yellow pigment is associated with follicular activity (DANNEEL 1947; CLEFFMANN 1953; GALBRAITH 1964). CLEFFMANN (1963) has proposed that an elevated mitotic rate in the follicular matrix is associated with banding in agouti animals and this view would seem to be supported by the work of GALBRAITH (1964) who demonstrated that the agouti pigment pattern could be altered by the administration of the mitotic inhibitor colchicine during the period of yellow band formation.

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The purpose of this work is to determine whether or not there is a relationship between patterns of mitotic activity in the hair bulb and expression of alleles at the agouti locus. This paper reports the results obtained in a study of the relationship between expression of the A^y , A , and a agouti-locus alleles and mitotic activity of the hair bulb.

MATERIALS AND METHODS

Agouti mice of strain C3Hf/HeHa and a yellow stock, derived from a line maintained by HERMAN B. CHASE, Brown University, which was segregating for the non-agouti black allele (a) were used. In this laboratory, the yellow stock was maintained by $A^y/a \times a/a$ brother-sister matings and by outcrossing the yellow animals to C57BL/Ch mice (a/a).

To insure that all hairs were in the same stage of hair growth at the time of sampling, resting hairs were plucked from the mid-dorsum at 24 days and again at 50 days after birth thereby stimulating a synchronous cycle of hair growth. Biopsies were taken four days (before the onset of pheomelanin synthesis in agouti animals) through 11 days after the second plucking (pheomelanin synthesis has ceased in virtually all agouti follicles by this time). The skin was fixed either in Bouin's fixative or AFA, serially sectioned at 10μ in a plane parallel to the long axis of the follicle, and stained with Harris' hematoxylin and eosin.

Agouti animals of a single litter were sacrificed serially (one on each day). Fifty agouti mice were used and 60 hair bulbs were scored in each animal. Genetically yellow and genetically black mice of the same sex within a litter were sacrificed at the same time, however, so that a direct comparison could be made between the follicles of yellow and black littermates in the same stage of hair growth. For each stage of hair growth, 20 zigzag hair bulbs from each of six yellow and six black animals, equally divided according to sex, were scored. An exception is among Anagen VI day 8 animals in which three yellow and three black mice, all females, were scored.

Each bulb scored was followed serially and thus the total number of mitotic figures throughout the entire hair bulb was counted. The follicles were classified according to their Anagen stage of hair growth, the type of pigment they were producing, and the type of hair they were producing. A word of explanation is required with regard to the latter classification. The pelage of the mouse is composed of four major hair types. The underfur is made up of zigzags and there are in addition three types of overhairs, the auchenes, awls, and monotrachs, all of which are longer than the zigzags. In a previous study (GALBRAITH 1969), it was found that the follicles which give rise to these different hair types can be separated into different classes according to the diameter of their hair bulb. This system of classification is used here (Table 1) but with the following modifications. The original classification was of fresh, living follicles, the one here of fixed tissue. As a consequence of tissue shrinkage due to fixation, the bulb diameters are smaller for each category than in unfixed tissue. Nevertheless, shrinkage is rather uniform, and in Anagen V and Anagen VI, the various classes are easily identified. In Anagen III and Anagen IV, however, the follicles have not attained their mature size and it is difficult to make class designations according to the original scheme. Since different sizes of bulbs nevertheless are

TABLE 1

Classification of hair follicle types according to bulb diameter in fixed tissue

Stage of growth	Zigzags		Cl. 1	Awls		Cl. 4	Cl. 5	Monotrachs
	Cl. 1	Cl. 2		Cl. 2	Cl. 3			
Anagen III	30-39 μ	40-49 μ	50-59 μ	60-69 μ	70-79 μ	80-89 μ	80-89 μ	80-89 μ
Anagen IV	40-49 μ	50-59 μ	60-69 μ	70-79 μ	80-89 μ	90-99 μ	90-99 μ	100 μ
Anagen V & VI	40-49 μ	50-59 μ	60-69 μ	70-79 μ	80-89 μ	90-99 μ	100-109 μ	110 μ

Abbreviation: Cl. = class.

present during these early stages of hair growth, class assignments were made but may be somewhat arbitrary with respect to the ultimate fate of these follicles at maturity. The only exception is the zigzags which can be identified on the basis of morphological criteria as well as bulb size. Data for agouti auchenes are not included in this report since this hair type is rare in the strain of animals used (GALBRAITH 1964, 1969). All bulbs were measured with a calibrated ocular micrometer and studies were performed with a bright field microscope at $500\times$.

The mean and the standard error of the number of dividing cells in A^y/a and a/a zigzag bulbs and in each category of agouti hair bulb according to type and class, pigmentation, and stage of growth was computed. Since there were no statistically significant differences between sexes, these data are grouped. There were also no statistically significant differences between Class 1 and Class 2 zigzags, so information from these groups is treated together under the heading "zigzags." Similarly, in agouti animals, Class 4 and Class 5 awls are treated as a single group since there were no statistically significant differences between them. The Student-Fisher small-sample t-test was used as a test of statistical significance. In addition, the significance of differences in mitotic rate between yellow and black littermates of the same sex was tested by the method of unpaired replicates.

RESULTS

Agouti animals (A/A): A comparison of the average mitotic indices in agouti hair bulbs which contain black pigment, yellow pigment, or a mixture of both black and yellow pigments is found in Table 2. The latter group of follicles is that which was in transition between the production of the two pigments and is designated as T1 in the case of those which were switching from the synthesis of black to yellow pigment, and T2 in the case of those which were completing the yellow phase and entering the second phase of eumelanin synthesis. It can be seen that in general, as a bulb moves out of the initial phase of eumelanin synthesis, into the T1 period, and then into the yellow phase, there is an increase in the mitotic activity of the follicle matrix. The rate of cell division continues to increase during the T2 phase and remains at a high level during the second period of eumelanin synthesis (days 9, 10, and 11 after plucking).

Figures 1 and 2 show a graphic comparison of the pattern of mitotic activity in eumelanic and phaeomelanic bulbs during the course of follicle growth and differentiation from Anagen III through the 11th day after plucking during Anagen VI. It is evident that during Anagen IV there is a distinct burst of mitotic activity in all bulb matrices regardless of hair type, class, or pigmentation. In the smaller hair bulbs, viz., the zigzags and Class 1 and 2 awls, the mitotic rate remains stable through Anagen V and then steadily accelerates throughout the first four days of Anagen VI. The rate of cell division in the bulbs of the larger awls, however, does not level off during Anagen V, but constantly increases throughout the period studied until the initial days of Anagen VI when there is a slight and temporary decrease in activity (Table 2). It is also evident that with each increment in bulb size according to class, there is a corresponding increase in the number of mitoses in the follicle matrix.

These figures also show that the average mitotic rate of phaeomelanic bulbs is generally higher than that of eumelanic bulbs. According to the small-sample t-test, these differences are statistically significant ($P < .01$) only in zigzags during Anagen IV and days 8 and 9 of Anagen VI and in Class 2 awls during Anagen

FIGURES 1 and 2.—Average number of dividing cells with 95% confidence intervals in agouti (A/A) hair bulbs in both the black and yellow phases. Points representing data from less than ten bulbs are omitted. Days noted under Anagen VI represent days elapsed after initiation of hair growth. Zigzags are in the first phase of eumelanin synthesis during Anagen III and IV and enter the second phase of eumelanin synthesis eight days after plucking. Most awls in which there is banding enter the second phase of eumelanin synthesis approximately ten days after plucking.

TABLE 2

Average number of dividing cells $\pm$ standard error per agouti hair bulb for all hair classes*

Hair type	III	IV	Anagen stage of hair growth				
			V	VI 8	9	10	11 days
Zigzags							
B	4.4 $\pm$ 0.2 (183)	6.5 $\pm$ 0.2 (119)	6.5 $\pm$ 3.5 (2)	5.8 $\pm$ 0.6 (30)	8.0 $\pm$ 0.3 (159)	8.5 $\pm$ 0.3 (137)	10.9 $\pm$ 0.3 (173)
T1	..	6.9 $\pm$ 0.3 (109)	8.6 $\pm$ 0.7 (16)	8.0 $\pm$ 0.0 (1)	..	..	..
Y	..	8.8 $\pm$ 0.3 (125)	9.1 $\pm$ 0.3 (153)	10.4 $\pm$ 0.4 (69)	9.6 $\pm$ 0.5 (35)	6.0 $\pm$ 0.0 (1)	14.0 $\pm$ 6.0 (2)
T2	..	..	..	10.5 $\pm$ 0.6 (40)	8.6 $\pm$ 0.6 (38)	..	..
Awls							
Class 1							
B	5.7 $\pm$ 0.3 (192)	9.1 $\pm$ 0.5 (53)	6.0 $\pm$ 3.0 (3)	..	8.7 $\pm$ 1.0 (6)	12.4 $\pm$ 0.8 (31)	11.7 $\pm$ 0.6 (24)
T1	..	11.4 $\pm$ 0.7 (40)	11.5 $\pm$ 1.1 (8)	11.0 $\pm$ 0.0 (1)	..	..	..
Y	..	11.1 $\pm$ 0.6 (35)	11.7 $\pm$ 0.6 (53)	14.9 $\pm$ 0.9 (3)	13.2 $\pm$ 1.5 (16)	10.0 $\pm$ 0.0 (1)	..
T2	..	..	..	18.0 $\pm$ 8.0 (2)	8.9 $\pm$ 1.2 (8)	19.2 $\pm$ 3.7 (5)	17.0 $\pm$ 0.0 (1)
Class 2							
B	8.1 $\pm$ 0.5 (82)	13.1 $\pm$ 0.5 (81)	12.9 $\pm$ 1.0 (28)	15.2 $\pm$ 1.7 (10)	19.5 $\pm$ 2.7 (4)	15.6 $\pm$ 1.1 (23)	19.3 $\pm$ 0.8 (45)
T1	..	13.3 $\pm$ 1.8 (15)	14.4 $\pm$ 2.0 (7)	18.2 $\pm$ 5.4 (4)	19.0 $\pm$ 2.0 (2)	..	..
Y	..	15.4 $\pm$ 1.3 (13)	16.2 $\pm$ 0.8 (29)	18.8 $\pm$ 1.0 (37)	18.3 $\pm$ 0.6 (66)	19.2 $\pm$ 1.4 (6)	15.2 $\pm$ 1.5 (4)
T2	..	..	..	..	23.2 $\pm$ 2.7 (5)	20.7 $\pm$ 2.1 (11)	21.2 $\pm$ 2.3 (8)
Awls							
Class 3							
B	9.5 $\pm$ 1.1 (13)	15.3 $\pm$ 0.6 (35)	16.8 $\pm$ 0.9 (31)	20.0 $\pm$ 1.1 (23)	23.4 $\pm$ 1.4 (24)	21.2 $\pm$ 1.6 (25)	21.7 $\pm$ 1.0 (36)
T1	..	..	15.5 $\pm$ 1.5 (2)	..	23.0 $\pm$ 2.3 (5)	27.0 $\pm$ 0.0 (1)	..
Y	..	18.0 $\pm$ 0.0 (1)	16.0 $\pm$ 2.1 (3)	22.6 $\pm$ 1.4 (17)	20.3 $\pm$ 1.0 (40)	23.8 $\pm$ 2.1 (5)	..
T2	..	..	..	..	30.2 $\pm$ 3.0 (5)	22.2 $\pm$ 1.4 (20)	23.2 $\pm$ 3.8 (4)
Class 4-5							
B	12.5 $\pm$ 1.2 $\ddagger$ (6)	17.0 $\pm$ 1.6 (17)	18.7 $\pm$ 1.4 (22)	25.5 $\pm$ 1.6 (25)	24.1 $\pm$ 1.0 (53)	26.9 $\pm$ 1.4 (27)	27.5 $\pm$ 0.9 (49)
T1	..	..	..	..	20.0 $\pm$ 0.0 (1)	..	..
Y	..	..	..	29.0 $\pm$ 4.0 (2)	23.1 $\pm$ 2.7 (7)	..	29.0 $\pm$ 0.0 (1)
T2	..	..	..	..	..	27.5 $\pm$ 2.5 (2)	..

TABLE 2

Average number of dividing cells $\pm$ standard error per agouti hair bulb for all hair classes*

Hair type	Anagen stage of hair growth						
	III	IV	V	$\frac{1}{2}$ VI 8	9	10	11 days
Monotrichs§							
B	12.5 $\pm$ 1.2‡ (6)	26.2 $\pm$ 3.8 (4)	26.0 $\pm$ 0.0 (1)	28.0 $\pm$ 5.0 (2)	31.7 $\pm$ 3.2 (3)	40.1 $\pm$ 4.7 (3)	33.0 $\pm$ 3.0 (7)

* Number of bulbs scored is in parentheses.
† Days noted in Anagen VI represent days after initiation of hair growth.
‡ Anagen III Class 4-5 awls and monotrichs could not be distinguished from each other and are therefore treated as a single category of hair type during this stage of hair growth.
§ Monotrichs produced only black pigment. Abbreviations: The type of pigment present in a bulb at the time of scoring is noted as follows: B=black; Y=yellow; T1=transition between initial black phase and yellow; T2=transition between yellow phase and second black phase.

FIGURE 3. Left—Histograms showing the number of dividing cells found among agouti (A/A) hair bulbs in the yellow phase and in the black phase during Anagen IV. Yellow bulbs had a significantly ($P < .01$) higher mitotic index than black bulbs at this stage.

Right—Histograms showing the number of dividing cells in genetically yellow (A^y/a) and genetically black (a/a) hair bulbs during Anagen V. Yellow bulbs had a significantly ($P < .01$) higher mitotic index than black bulbs at this stage.

V. All other comparisons of mitotic rate between yellow and black bulbs revealed no statistically significant differences.

Figure 3 illustrates the frequency distributions of dividing cells in yellow and black agouti zigzag bulbs whose means differ significantly. It can be seen that the modal class of yellow bulbs has a larger number of mitoses than the modal class of black bulbs, but that the range of variation is similar in both phaeomelanin and eumelanin bulbs. It seems unlikely that these two distributions represent different populations of bulbs according to the rate of cell division. Frequency distributions of bulbs which did not differ significantly, of course, are even more similar than those depicted here.

It should be pointed out that dividing nuclei of matrix cells adjacent to the zone of the hair bulb in which the melanocytes reside are less readily detected when the dense, opaque black pigment granules are present than when the more translucent phaeomelanin granules are present. As a consequence, the few dividing cells which occur in this area of the bulb are more likely to be detected in yellow than in black bulbs. This source of error could have affected the results so that the black bulbs may actually have a slightly higher mitotic index than this study indicates.

Genetically yellow (A^y/a) and genetically black (a/a) animals: As can be seen in Figure 4, yellow and black bulb matrices have approximately the same

FIGURE 4.—Average number of dividing cells with 95% confidence intervals in genetically yellow (A^y/a) and genetically black (a/a) hair bulbs. Days noted under Anagen VI represent days elapsed after initiation of hair growth.

patterns of mitotic activity as agouti hair bulbs. There is a burst of mitotic activity during Anagen IV which, however, unlike agouti zigzags, continues into Anagen V. There is then a slight diminution or leveling off of mitotic activity and this is followed by an acceleration of activity during days 10 and 11 of hair growth.

Figure 4 reveals that there is little difference between genetically yellow and genetically black hair bulbs with respect either to the pattern of mitotic activity during the growth period studied or to the mitotic index of a given growth stage. According to the small-sample t-test, yellow bulbs had a statistically significant ($P < .01$) higher mitotic index than black bulbs only in Anagen V and Anagen VI day 10. Comparisons between 39 yellow and black littermates by the method of unpaired replicates revealed only two pairs, one female pair in Anagen VI day 9, and one male pair in Anagen VI day 10, in which yellow bulbs had a significantly ($P < .01$) higher mitotic rate than black bulbs. In two other pairs, both Anagen VI day 11 males, the mitotic rate of black bulbs was significantly ($P < .01$) higher than that of yellow bulbs. In looking for trends, regardless of statistically significant differences, it was found that of the 39 comparisons made, the average mitotic count (rounded off to whole numbers) of yellow bulbs exceeded that of black bulbs in 17 cases and 14 pairs were identical. Thus, yellow bulbs had a higher mitotic index in less than half (about 44%) of the cases. This would seem to represent a slight but probably insignificant trend toward an increased rate of cell division in yellow bulbs.

Figure 3 illustrates frequency distributions of genetically yellow and black bulbs which had significantly different mitotic averages. It can be seen that these distributions are similar to those of agouti bulbs in that the modal class of yellow bulbs has a higher mitotic index than that of black bulbs, but that both have a similar range of variation.

DISCUSSION

The work of SILVERS and RUSSELL (1955) and SILVERS (1958a,b) clearly demonstrates that agouti-locus alleles do not act autonomously within melanocytes, but act by altering the follicular environment in which pigment cells reside, thereby creating appropriate conditions for the synthesis of phaeomelanin or eumelanin. This report demonstrates that agouti-locus alleles do not alter the follicular environment by acting as regulators of mitotic activity within the hair bulb.

This conclusion contradicts that of CLEFFMANN (1963) who states that in agouti mice "one finds a maximum of mitotic activity . . . [which] corresponds to the time of yellow pigment formation." CLEFFMANN's studies, however, were of neonatal follicles (of unspecified bulb mass) whose growth and differentiation is asynchronous (cf. MANN 1962). Furthermore, it is not clear whether his data represent mitotic indices of yellow follicles only or a mixture of yellow and black follicles, both of which are present during the banding period. The present study has controlled these variables and also has made direct comparisons between genetically yellow and genetically black follicles. Therefore, it is felt that this

report more accurately portrays the mitotic patterns of phaeomelanin and eumelanin follicles than does CLEFFMANN'S.

Initiation of banding in most agouti hairs occurs during Anagen IV when the mitotic rate begins to increase and the follicles are in transition from the period of growth and development to the phase of rapid hair production. The existence of a direct causal relationship between these two events however, is unlikely for the following reasons. First, black bulbs, as well as yellow bulbs, have an increased mitotic rate in Anagen IV. The increase in black bulbs is slightly lower than in yellow bulbs but, since it is unlikely that all follicles are in precisely the same stage of development at any given time, the black bulbs probably represent that portion of the follicle population which is on the trailing edge of the developmental schedule. Secondly, if there is a causal relationship between increased mitotic activity and phaeomelanin synthesis, one would expect the second phase of eumelanin synthesis in agouti bulbs to be accompanied by a decreased mitotic rate. It is clear that this does not occur. Indeed, the rate of cell division during the return to synthesis of black pigment is, in most cases, higher than during band formation. Finally, the virtually identical mitotic rates of genetically yellow and genetically black bulbs at all stages of hair growth would argue against a relationship between mitotic activity and the quality of pigment synthesized.

If there is a direct relation between mitotic activity in the hair bulb and the manufacture of yellow pigment, there should be a critical number of dividing cells above which the synthesis of phaeomelanin is switched on. But what is that number? In view of the very similar distribution of numbers of dividing cells in both agouti bulbs in the black and yellow phase and in genetically black and genetically yellow bulbs, such a figure is impossible to discern. It is especially noteworthy that A^y/a , A/A , and a/a bulbs in Anagen III have virtually the same low mitotic index; yet yellow pigment is nevertheless produced only by the A^y/a genotype. Also, all three genotypes have the same burst of mitotic activity in Anagen IV, but the melanogenic activity of only the A/A genotype is affected. Note as well that the agouti monotrichs, class 5 awls, and possibly the class 4 awls, do not synthesize yellow pigment (GALBRAITH 1969), yet have the greatest rate of mitotic activity. The difference between these bulbs and those of other bulb classes which do manufacture yellow pigment might lie in the ratio of dividing to nondividing cells. But this explanation would have to remain as a special case since it does not account for either the production of both banded and nonbanded hairs by agouti bulbs of similar volume, nor the production of different pigments by A^y/a and a/a bulbs of identical mass.

Thus, the mechanism by which agouti-locus alleles act to condition the follicular environment and influence melanocyte function remains obscure. Metabolic studies, such as those conducted by CLEFFMANN (1964) and by HOLSTEIN, BURNETT and QUEVEDO (1967) would seem to offer the most promise in understanding the action of genes at this locus.

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SUMMARY

Comparisons of mitotic activity in the bulbs of hair follicles during the course of follicle growth and differentiation from Anagen III through the first few days of Anagen VI of the hair growth cycle of A^y/a , A/A , and a/a mice were performed. It was found that the pattern of mitotic activity was similar in all three genotypes and that differences in mitotic rate probably do not account for either banding in agouti mice or for differences in pigmentation between genetically yellow and genetically black animals. It is suggested, therefore, that agouti-locus alleles act to alter the follicular environment by some means other than regulation of mitotic activity.

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