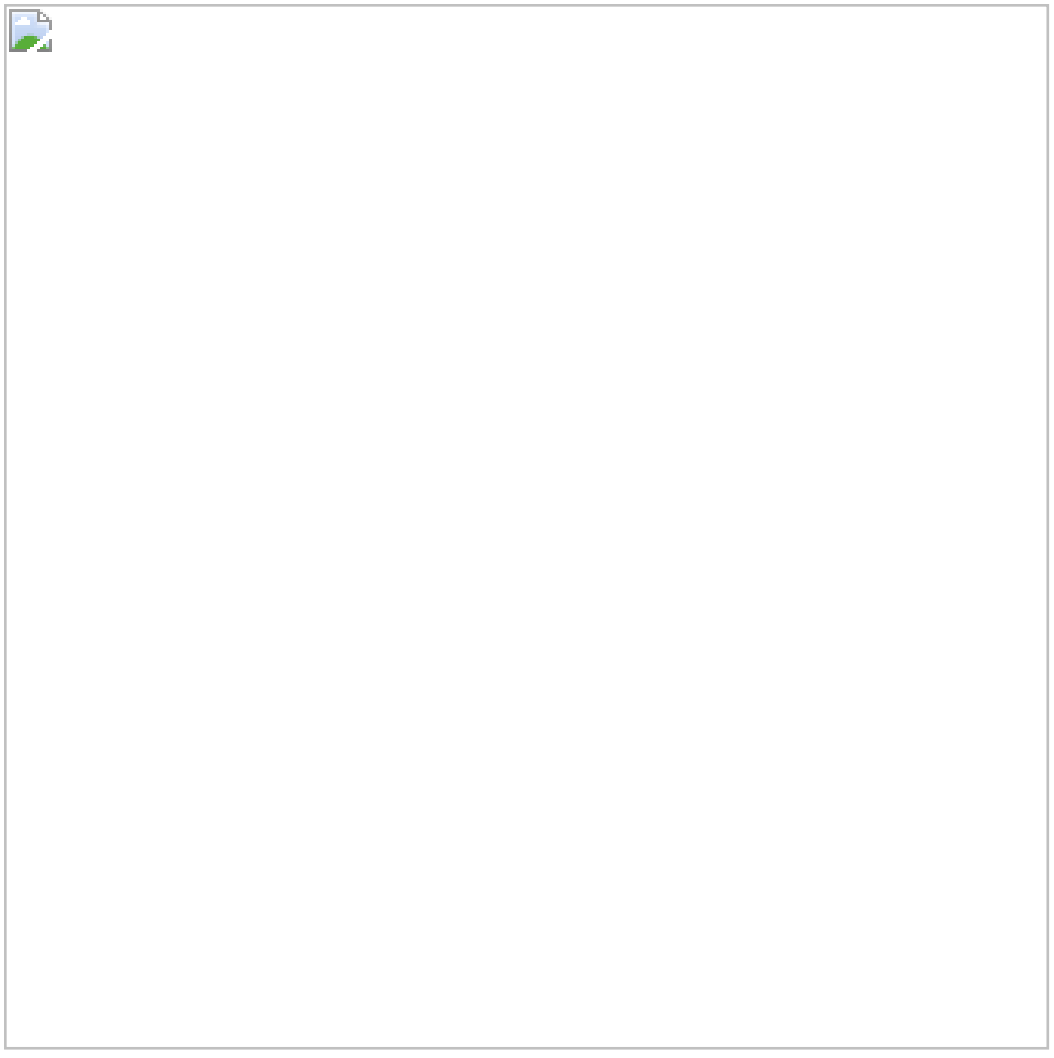

A Pleiotropic Gene which Controls Coat Color and Lethality in Early Development in the Mouse

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Source: Zoological Science, 12(6) : 675-681

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.12.675

ZOOLOGICAL SCIENCE 12: 675–681 (1995)

REVIEW

A Pleiotropic Gene which Controls Coat Color and Lethality in Early Development in the Mouse

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INTRODUCTION

Pleiotropic gene can be defined as a gene which controls multiple phenotypes. Melanocytes, originating from the neural crest and differentiating into a cell type which specifically produces melanin pigments in vertebrates, are involved in various types of pleiotropism. In the case of "white spotting", the genes controlling ligands or receptor for melanocyte differentiation also express in other cell types such as haemopoietic cells leading to anemia as well as white spotting. In the case of the albino mutant which possesses a deficiency in enzyme tyrosinase and lacks melanin production in the melanocytes also expresses abnormality in the optic chiasm. On the other hand, a mutation at the agouti locus expresses yellow hair instead of agouti hair of the wild type mouse when heterozygous, and exhibits lethality in early development when homozygous.

In wild-type mice, melanocytes located in hair follicles produce black pigment, eumelanin, at the beginning of hair growth. They subsequently produce yellow pigment, pheomelanin, and finally produce eumelanin. Therefore, their hair is characterized by a subterminal band of yellow, with the rest of it showing black or brown color, since melanin granules (melanosomes) formed in melanocytes are constantly transferred to hair keratinocytes. This characteristic is called agouti pattern and is known to be controlled by the *A* allele, the wild type allele at the *a* (agouti) locus.

Both eumelanin and pheomelanin are known to be derived from a common precursor, tyrosine, which is converted to dopa (3,4-dihydroxyphenylalanine) and subsequently to dopaquinone by the action of tyrosinase (Fig. 1). The enzyme is synthesized and functions only in melanocytes and is considered the key enzyme in melanogenesis. Eumelanin is formed through polymerization of indole-5,6-quinone or indole-5,6-guinone-2-carboxylic acid derived from dopaquinone via leucodopachrome [18, 28]. Pheomelanin is a polymer of 5-S-cysteinyldopa and 2-S-cysteinyldopa derived from dopaquinone [32]. On the other hand, melanin is formed in a specific organelle, melanosome, which develops as a conjugated structure between ER-derived vesicles and microvesicles from GERL (Golgi-associated-ER-lysosomes) [1, 15, 25, 43]. Melanosomes containing eumelanin are called eumelanosomes and are characterized by a striated longitudinal matrix in their ovoid-shaped envelope, while those containing pheomelanin are designated pheomelanosomes, and are characterized by their spherical-shaped multivesicular structures [15, 25, 36].

Expression of agouti pattern formation is altered by genetic substitutions at the *a* locus and the *e* (extension) locus. Animals heterozygous for A^y (lethal yellow) allele (A^y/A or A^y/a) exhibit yellow coat color in addition to obesity and susceptibility to cancer, while animals homozygous for *a* allele produce solely black hair except on their ear and tail (Table 1). On the other hand, homozygous A^y/A^y embryos display characteristic abnormalities at the blastocyst stage and die before implantation [6, 7, 35]. There are some other alleles at the *a* locus which exhibit different phenotypes depending on the area of the body. These observations indicate that the *a* locus controls a mechanism which determines the type of melanin synthesized in the melanocytes. Mice homozygous for the *e* (recessive yellow) allele at the *e* locus also exhibit yellow coat color which is not distinguishable from the lethal yellow though the gene acts autonomously within the melanocytes [19, 31]. Questions arise as to how the agouti gene, one of the major coat color genes, is involved in the pleiotropic lethality in early development and how it is related to the action of the *e* locus.

SITE OF THE GENE ACTION

As to the mode of function of agouti locus, Silvers and Russell have clearly demonstrated that the genes at the *a* locus act through the follicular environment [38]. They grafted intensely pigmented black mice of the genotype a/a, C/c^c shortly after birth with neonatal skin from albino mice of the genotype A^y/a, c^e/c^e, and found some intensely colored

Received August 7, 1995

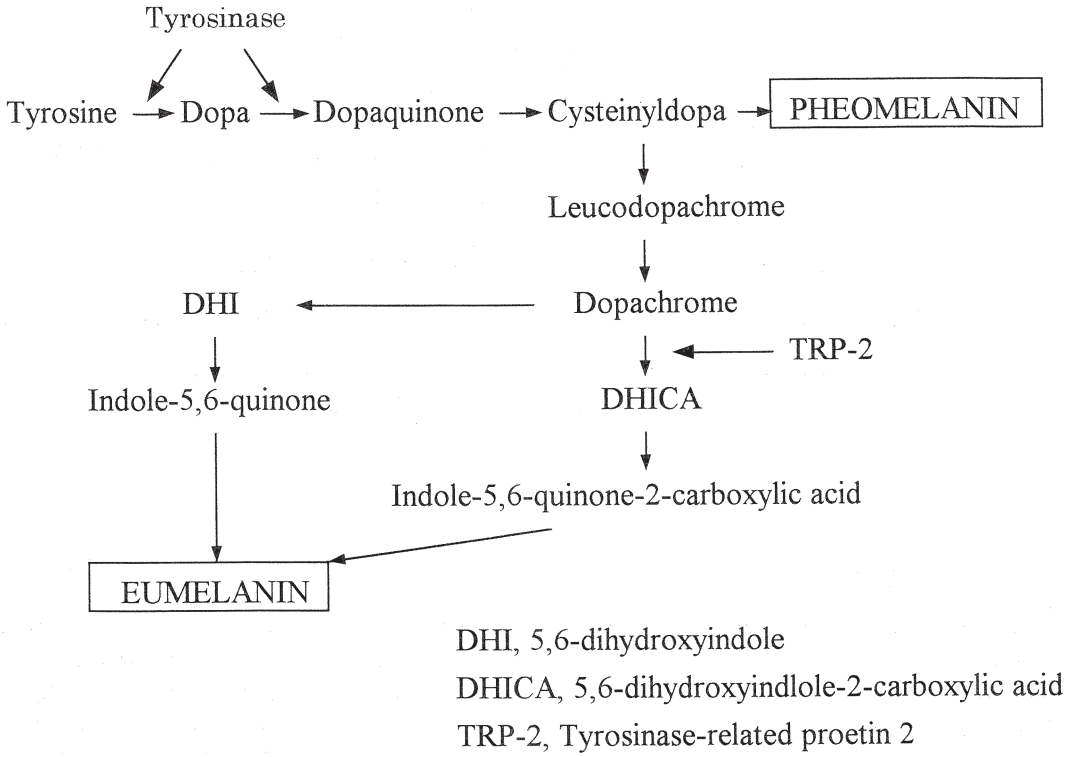

FIG. 1. Melanogeneic pathway. Modified from Kobayashi *et al.* [18].

TABLE 1. Representative alleles at the *a* and *e* loci

locus	alleles	nomenclature	phenotype
a	A	wild type	agouti hair pattern
	A^{y}	lethal yellow	lethal when homozygous heterozygotes exhibit yellow hair, obesity, tumor susceptivility, diabetes
	A^{vy}	viable yellow	similar to a^{y}, but not lethal
	a	nonagouti	black hair
	a^{t}	black-and-tan	black dorsal hair, yellow ventral hair
e	E	wild type	when $A/-$ exhibits agouti hair
	E^{so}	sombre	yellow hair on sides and perineum
	E^{tob}	tobacco	black until 8 weeks, then agouti flanks
	e	recessive yellow	yellow hair

yellow hairs among the non-pigmented hairs within the border of the graft. In this particular transplantation, melanocytes of the host which were originally synthesizing eumelanin, invaded the grafted skin which possessed a lethal yellow gene and began pheomelanogenesis. Similar results were obtained in reciprocal transplantations. These observations led them to hypothesize that the kind of melanin synthesized by the invading cells is not determined by the genotype of melanocytes but by the genetic constitution of the receiving hair follicles and that the genes at the agouti locus produce the effect by determining follicular milieu.

Sakurai *et al.* have also provided evidence to support Silvers and Russel's hypothesis: an electronmicroscopic observation on hair follicles revealed the presence of the two types of melanosomes, eumelanosomes and pheomelanosomes, in the transitional phase in agouti pattern formation [36]. This result indicates that the shift between the two pathways of melanin formation occurs within each single cell. The possibility of differentiation of two cell types or migration of particular melanocytes can be excluded.

Extensive studies to pinpoint the site of the action of the *a* locus have been carried out by recombination experiments between epidermis and dermis with different genotypes [22, 30]. Results of this line of research have suggested that the site of the gene action of the *a* locus is the dermis probably of the hair follicle. In some experiments, however, A^{y} allele

was shown to act via epidermal as well as dermal components of the skin to induce pheomelanin formation. It must be noted, however, that, in contrast to the case of genes at the *a* locus, *e* gene acts autonomously within melanocytes [14, 37, 38].

THE EFFECT OF MSH ON PHEOMELANOGENESIS

Geschwind and Huseby found that a single hybrid mouse fed with a diet containing tilbestrol for 11 months in order to produce mammary tumor developed much darker hair than normal [11]. At necropsy they found a pituitary tumor; microscopic observation of dorsal hair from the darkened animal revealed that the subterminal yellow band had been obliterated. Based on the assumption that the darkening was induced by α-MSH (melanocyte stimulating hormone), they injected mice of various genotypes with α-MSH, and found alteration of the agouti pattern. The striking effect they found was the conversion of pheomelanic type to that of eumelanic [12, 13]. In the A^y/a mouse whose hair had been plucked 5–8 days prior to the treatment, only intensely black hair emerged. In contrast, MSH had no effect on animals homozygous for the brown (*b*), dilute (*d*) or pink eyed dilution (*p*). Besides, coloration of the recessive yellow (*e*/*e*) was completely unaltered by MSH administration. Geschwind and his colleague had emphasized the relationship between tyrosinase activity and fine structure of melanosome, and also between types of melanin formed in melanocyte and tyrosinase activity, i.e. maximum tyrosinase activity upon a highly organized melanosome structure results in eumelanin formation. They did not argue, however, about the precise mechanism of α-MSH in the conversion of melanogenesis.

In order to elucidate the interaction between α-MSH and the A^y gene, we examined the effect of α-MSH on the hair-follicle melanocytes of lethal yellow (A^y/a) in organ culture [40]. In the control culture, pheomelanin was observed in most of the hair follicles. In contrast, the hair follicles of the α-MSH-treated explants were found to contain eumelanin. The frequency of hair follicles with eumelanin markedly increased within 12 hr after the α-MSH addition, and reached a maximum of approximately 95% at 48 hr. Our electron microscopic observations demonstrated that the shift in the color of the pigments produced was coupled with the shift in the fine structure of melanosomes. In the hair-follicle melanocytes of the α-MSH treated explants, eumelanosomes, in addition to mature pheomelanosomes, were found together with irregular melanosomes which were considered to be mosaic type melanosomes consisting of eumelanic and pheomelanic components. Therefore, it seemed probable that the shift from pheomelanogenesis to eumelanogenesis occurred in each melanosome in the process of forming and depositing melanin. This observation also leads us to assume that the structure of melanosomes is not predetermined by the gene product of the *a* locus. Eumelanin formation was also induced in the hair-follicle melanocytes of the lethal yellow by treating the culture with c-AMP (cyclic adenosine monophosphate) or dbcAMP (dibutyryl cAMP). This result seems to indicate that the induction of eumelanogenesis by α-MSH is mediated by cAMP in melanocytes. It is worth noting that the induction of eumelanin formation in the organ culture was blocked by either actinomycin D or cycloheximide treatment, suggesting the involvement of transcription and translation in the hormonal induction. Therefore, we assumed that the product of the agouti locus exerts its action by interacting with α-MSH-cAMP system and that excess amount of α-MSH probably antagonizes the product at the α-MSH receptor.

Attention was also focused on the response to α-MSH of another pheomelanic mutant, *e*/*e*; we cultured skin of newborn mouse of the recessive yellow and examined the effect of α-MSH and dbcAMP [41]. In contrast to the case of the lethal yellow, eumelanogenesis was not induced by α-MSH, whereas the skin explants exposed to DbcAMP were found to contain eumelanin (Fig. 2). The results of our *in vitro*

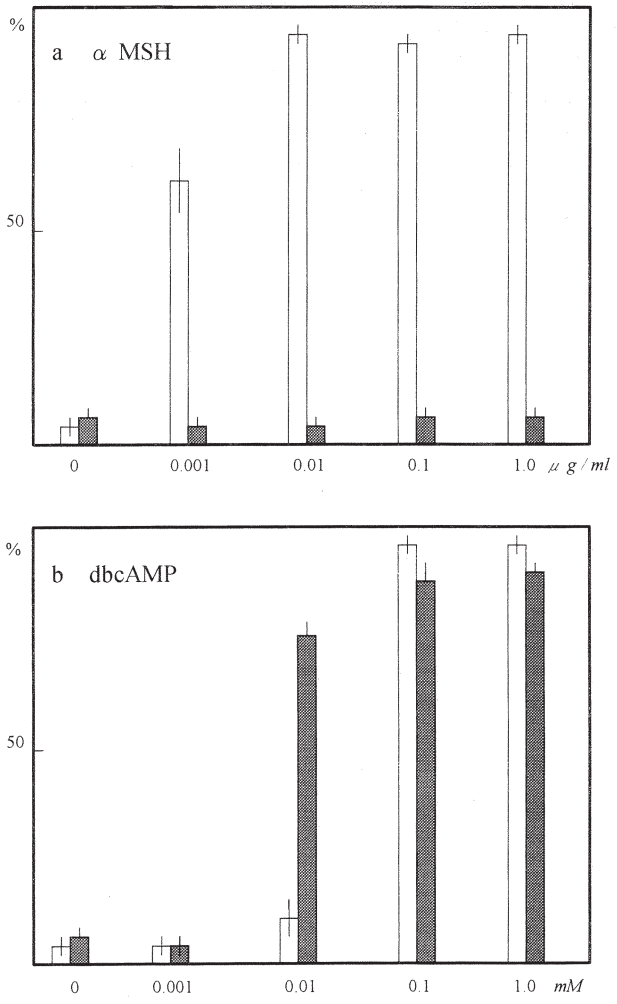

FIG. 2. Effects of α-MSH and dibutyryl cyclic AMP (dbcAMP) on the induction of eumelanin synthesis *in vitro*. Frequency (percent) = the number of hair bulbs with eumelanin/total number of hair bulbs in each explant ×100. Open columns represent A^y/a; shaded columns represent *e*/*e*. Bars indicate standard errors. a, α-MSH; b, dbcAMP.

studies seem to indicate that the action of α-MSH on the lethal-yellow melanocyte is mediated by cAMP as the second messenger and that the melanocyte of the recessive-yellow mice has a deficiency in responding to α-MSH, though it is primarily capable of producing eumelanin. In other words, the *e* locus seems to control the functionality of the α-MSH receptor.

Based on the hypothesis that the *e* locus controls a mechanism involved in the α-MSH receptor, Takeuchi *et al.* examined the effect of forskolin which is a potent activator of adenylate cyclase in the membrane, and choleratoxin which is an activator of G-protein on pheomelanogenesis [39]. Melanocytes of the lethal yellow mice were induced to form eumelanin by the agents, whereas in the recessive yellow mice, hair-follicle melanocytes formed only pheomelanin. Therefore, it is likely that melanocytes with the genotype e/e exhibit defect in the function of α-MSH-adenylyl cyclase system. They then proposed that the α-MSH receptor is involved in the shift between eumelanogenesis and pheomelanogenesis; when follicular cells excert the products of A^y or A, they block the MSH receptor, resulting in a decrease in cAMP level in the cells. However, the gene product, agouti factor or agouti protein, can be removed by an excess amount of α-MSH to increase the cAMP level in the cells. Melanocytes of the recessive yellow mice continue to produce pheomelanin because they have a deficiency in a function of α-MSH receptor - adenylyl cyclase system.

LETHAL EFFECT OF A^y ALLELE

Since as early as in 1908 Cuenót, and in 1910 Castle and Little identified the mutant, A^y as prenatal homozygous lethal, a number of investigators have analyzed this mutatation *in vitro* as well as *in vivo* [6, 7]. It is known that embryos homozygous for A^y are arrested *in utero* as partially implanted blastocysts which contain abnormal trophoblastic giant cells [9, 10, 17, 34]. Several abnormalities have been reported as to the appearance of abnormalities at the preimplantation stage in embryos homozygous for A^y. It has been reported that A^y homozygotes possessed the blastomeres that were arrested as early as at the third cleavage stage and were observed in compacted morula and blastocysts [5, 29]. Other defects such as retardation of cleavage and hatching have also been observed [16, 29]. Some investigators, however, reported that no distinct defect was seen during blastocyst formation in the A^y/A^y embryo [27, 34]. Papaioannou in her *in vitro* observation reported that the failure of hatching in embryos from A^y/a x A^y/a cross of a inbred strain was not detected in a 24-hour-culture [25].

Papaioannou and Gardner approached the problems to determine which specific tissue the A^y gene is responsible for, by blastocyst injection of chimera between embryos from A^y/a^e x A^y/a^e and a^e/a^e x a^e/a^e crosses [27]. The inner cell mass (ICM) derived from A^y homozygotes seemed to be rescued by chimeric environment but the trophectoderm (TE) was not. They concluded that the defect of A^y homozygotes occurred in only TE and was cell-autonomous. Later, Papaioannou examined the potency of ICM and TE to proliferate in culture condition with or without fibroblast feeder layer [26]. No rescue of either tisssues from A^y homozygotes was shown; the result suggested that the gene affected both tissues of the late blastocysts.

Recently Barsh and his co-workers investigated the lethality in A^y homozygotes by aggregation chimera between embryos from A^y/a x Ay/a and A/A x A/A crosses using a molecular probe at the Emv-15 locus that distinguishes among the three alleles A^y, A and a [3]. Their conclusion was that the cells originating in A^y homozygote were not capable of surviving until 9.5 dpc (days post coitum) in the chimeric environment and that the defect of A^y homozygote was cell autonomous.

We cultured embryos from the control cross (a/a x a/a) and the experimental cross (A^y/a x A^y/a) in order to examine the morphological abnormalities of A^y/A^y embryos and found no significant difference between the two crosses until blastocyst stage [35]. Large excluded cells were observed in only one case in each cross. They were as large as a blastomere of 16-cell stage embryos in blastocoel of early blastocysts. In these cases, however, we did not find any specific abnormalities. In order to examine the ability of cell division of the mutant embryos, 8-cell stage embryos were dissociated into individual blastomeres and the outcome of each cell was recorded. All embryos were found to develop into blastocysts with no retardation. These results seem to indicate that the arrested cells previously observed by some investigators were not a result of a specific defect of the A^y/A^y embryos, which developed normally until blastocyst formation.

In our study, the ability of hatching of A^y/A^y embryos was examined *in vitro*. In the experimental cross (A^y/a x A^y/a), the hatching ratio was 47.1% (32/68), whereas the ratio was 76.9% (40/52) in the control crosses (a/a x a/a). There was statistically significant difference between the two groups. The ratio between the control cross and the experimental cross was 4:2.4 indicating a possibility that the hatching ratio in heterozygote of A^y decreased. In the cross of A^y/a x a/a, the ratio was 79.3% (23/29) which was not significantly different from the cross of a/a x a/a, but significantly different from the cross of A^y/a x A^y/a. Thus, the decreased hatching ratio in the experimental cross appears to be caused by A^y homozygote. These results indicate that A^y/A^y fail to hatch *in vitro*.

To elucidate the mechanism of the defect, an attempt was made to investigate the factor which rescues the embryo from the defect. As one of the factors the effect of cAMP on hatching was examined: dbcAMP, an analogue of cAMP permeable to the membrane, was added to the culture medium at the concentration of 1 mM. The result is shown in Figure 3. In the control cross (a/a x a/a), when 1mM dbcAMP was added, 30 out of 38 blastocysts hatched. The hatching ratio, 78.9%, remained at the same level as with the case without dbcAMP (76.9%). In the experimental cross,

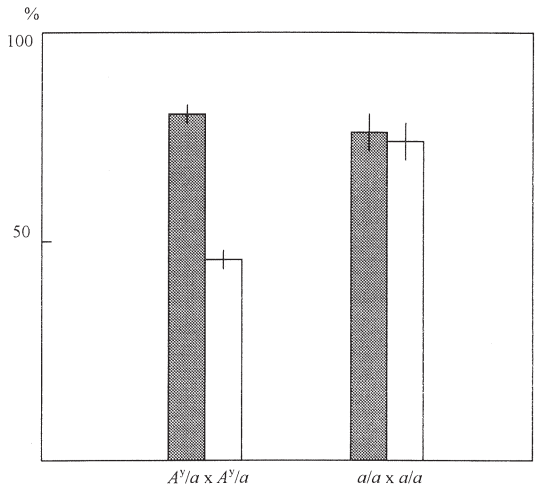

FIG. 3. Effect of dbcAMP on hatching of mouse embryos *in vitro*. Shaded columns represent dbcAMP-treated embryos; open columns represent untreated embryos. Bars indicate standard errors.

however, there was an increase in the ratio of hatching by addition of dbcAMP: 46 out of 56 blastocysts hatched. The percentage, 82.1%, was significantly greater when compared with the case without dbcAMP. The ratio was not different from the one in the control cross.

The result that adding dbcAMP to the culture medium gives rise to an increase in the hatching ratio of embryos from the experimental cross suggested that the cAMP level in A^y homozygotes was lower than that in normal embryos. We measured the cAMP level in blastocysts from the experimental and control crosses; the mean of 27 blastocysts from 4 litters was 3.2 ± 0.1 fmol in the control cross and 2.5 ± 0.1 fmol in the experimental cross. The blastocysts in the experimental cross possess significantly lower level of cAMP than those of the control cross.

Since it is conceivable that the lower level of cAMP in A^y homozygote is associated with the defect of adenylyl cyclase, we attempted to activate adenylyl cyclase by utilizing forskolin, cholera toxin and IBMX. The result showed that the ratio in the experimental cross was significantly increased by addition of the activators (68.5%) in comparison with no addition (47.1%,), whereas the ratio in the control cross was at the same level as without activators [35]. These results seem to suggest that A^yhomozygote embryos possess a defect in the signal transduction system mediated by adenylyl cyclase during hatching.

MOLECULAR STRUCTURES AND EXPRESSION OF THE *e* AND *a* LOCI

Our hypothesis that *e* locus controls functionality of MSH receptor (MSH-R) has been verified by cloning and sequencing the wild-type as well as the mutant alleles of the *e* locus. Robbins *et al.* reported that the coding sequence of the wild-type allele (E^+) consisted of a single 948 nt-exon protein presumably MSH-R, and that the recessive yellow (*e*) possessed a frameshift which produced a prematurely terminated protein presumably a nonfunctional receptor [33]. This was supported by transfection experiments. Cells transfected with the E^+ allele responded normally to α-MSH with a 2 to 5 fold increase in intracellular cAMP, while the *e* allele was unable to couple functionally to adenylyl cyclase, even at 0.1 μM concentraton of α-MSH. On the other hand, dominant extension alleles, E^{so}, E^{so-3J} and E^{tob}, were shown to possess base substitutions. The two sombre mutations occurred in extracellular half of the second transmembrane domain and have been shown to be consititutive and unresponsive to α-MSH, resulting in a dominant and epistatic phenotypes to even the most dominant alleles at the agouti locus, while E^{tob} allele encodes a hyperactive hormone-responsive receptor [33]. These molecular characteristics of the mutants agree with phenotypes of the corresponding extension alleles. Their results clearly indicate that the *e* locus controls the MSH-R.

On the other hand, Bultman *et al.* and Miller *et al.* [4, 24] cloned and characterized the mouse *a* locus and found that the wild type allele, *A*, consisted of 4 exons that encoded 131 amino acid protein. Using the first exon or portion of the first intron as a probe, they analyzed the structural alteration associated with *a* and a^t mutations. Each of these mutant alleles contained a structural alteration caused by the presence of extra DNA (at least 11 kb or 5 kb for *a* or a^t mutations, respectively) within the first intron of the wild type gene.

As to the expression of the agouti locus, the northern blot analysis showed that the wild-type allele, *A*, expressed 0.8 kb transcript in dorsal and ventral skin, whereas a^t/a^t expressed the transcript in ventral skin. In contrast, no 0.8 kb transcript was detected in a/a skin, while A^y is associated with a marked increase in the expression of a larger than normal transcript [42]. It was also shown by the northern analysis that expression of the wild-type allele is most enhanced on day-3 neonates, and coincide the agouti pattern formation in the wild-type hairs. These results appear to agree with the phenotypes of these alleles (Table 1).

Lu *et al.* demonstrated using patially purified recombinant agouti protein that the protein antagonize the action of α-MSH at the melanocortin receptor and blocks α-MSH stimulation of adenylyl cyclase [20]. The agouti protein also competes with adrenocorticotropic hormone (ACTH), which contains the α-MSH peptide. Miller *et al.* cloned the A^y/a^x genotype based on the fact that a^x or *a* alleles do not produce the transcripts except for the testes. The A^y cDNA represents a 923-nucleotide RNA in contrast to the 730-nucleotide of the *A* cDNA, but is completely identical to the *A* cDNA in the region of exon 2–4, which contain the 131-codon RF (Fig. 4). The A^y allele exhibits a striking contrast in the expression pattern with the wild type, where expression is restricted to testis and neonatal skin; $A^y/-$ animals overexpress the size-altered transcript in all the tissues examined including the brain, liver, lung, spleen and kidney as well as the skin [4, 24].

MOLECULAR MECHANISM OF THE LETHAL EFFECT OF A^y ALLELE

Although A^y allele is considered to be a single gene, the heterozygote exhibits obesity, increased tumor susceptibility and pheomelanin synthesis, while A^y/A^y homozygote expresses preimplantation embryonic death. It has been difficult to explain these pleiotropic effects including the lethal effect as the action of a single product of the A^y allele except for the pleiotropic effects in various tissues. A rearrangement in the regulatory region of the *a* locus might result in an constitutive expression [2]. In fact, the ubiquitous expression of the A^y gene has been demonstrated by the northern analysis using the A^y probes [4, 8, 24]. Evidence obtained by detecting restriction fragments using different probes has shown that the A^y mutation is caused by a 120 kb deletion which removes 5' flanking sequence of *agouti* exon 1A (Fig. 4).

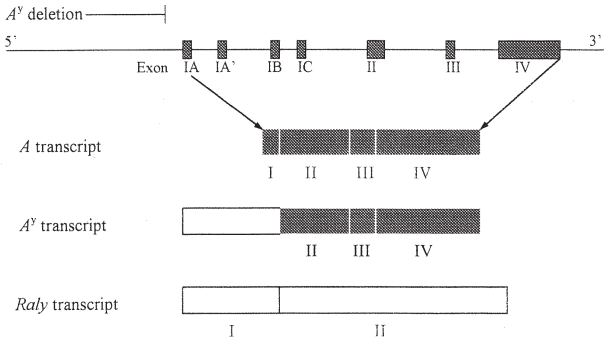

FIG. 4. Genomic structure of the *agouti* locus and the transcripts of the alleles, *A* and A^y, and *Raly* gene. Modified from Bultman *et al.* [4] and Duhl *et al.* [8].

Lethal effect of the A^y, however, can not be solely accounted by the deletion found in the regulatory region of the *a* locus. There might be another genetic factor involved in the early developmental stage of the mouse. Duhl *et al.* identified in *a*/*a* genotype a 296 amino acid open reading frame within the A^y deletion [8, 23]. The open reading frame did not hybridize to any A^y-specific fragment, but did detect the *a*-specific fragments, indicating that most or all of the open reading frame was contained in the 120 kb A^y deletion. This region has been shown to be highly similar to a human gene which encodes heterogeneous nuclear RNA-binding protein C and designated as *Raly* [8, 23]. The extra sequence found at the 5' end of the A^y mRNA was found to be derived from part of *Raly* open reading frame. Thus A^y mRNA is considered to be an alternatively spliced hybrid RNA which consists of most part of *Raly* and exon II, III and IV of the agouti gene. By using RT-PCR, presence of *Raly* RNA has been detected at relatively high levels in unfertilized eggs and relatively low levels in blastocysts. The importance of *Raly* in development has also been verified by inhibition of *Raly* expression in which 5×10^8 molecules of sense or antisense oligonucleotides were injected into 1-cell fertilized embryos. In the embryos transferred into the oviducts of pseudopregnant females, only 45% of those received the antisense oligonucleotide were recovered compared to 87% and 77% for the uninjected and sense controls, respectively. Nearly all of the embryos injected with the antisense oligonuleotide appeared morphologically abnormal at 3.5 dpc [8].

The deletion found in A^y allele extends from the 3' end of *Raly* to the 5' upstream region of exon I of the *a* locus [8, 23, 24]. As a consequence of the deletion, a fusion gene is reconstructed which is under the control of the *Raly* promoter. This also leads to an alternative splicing giving rise to a fused mRNA lacking exon I of the agouti locus [8, 23, 24] (Fig. 4). Therefore it is reasonable to assume that the products of *Raly* is responsible for the lethality of A^y allele for it seems non-functional in the A^y mutation and agrees with its recessive nature. It has been shown that the wild type *Raly* which encodes RNA-binding protein is expressed in early developmental stages as well as in adult life [8, 23] and that the antisense treatment blocks normal development [8]. Thus *Raly* is playing an indispensable role in development, probably in processing and protecting RNA transcripts. Etiology of the pleiotropic effects of A^y mutation is summarized in Figure 5.

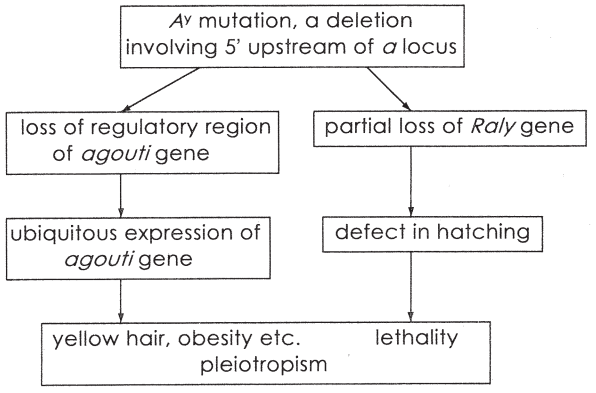

FIG. 5. The pleiotropic effects of A^y mutation.

On the other hand, the involvement of cAMP in the hatching of mouse embryo has also been reported; elevating cAMP level in morulae and blastocysts increases the rate of blastocoel expansion [21] and recovers the hatching ratio of A^y homozygotes [35], indicating that the adenylyl cyclase-mediated transduction system is ready to function before the implantation stage. It is, however, unlikely that overproduction of agouti protein that block α-MSH-R causes a recessive trait of lethality. Even if the agouti product blocks receptors of other ligands, it should produce a dominant trait. At present, a possibility that the hn RNA-binding protein is involved in generating adenylyl cyclase activity can not be excluded and should be a subject of future research.

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