Effects of the lethal yellow (A\textsuperscript{y}) mutation in mouse aggregation chimeras

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Summary

The \textit{Ay} allele is a recessive lethal mutation at the mouse agouti locus, which results in embryonic death around the time of implantation. In the heterozygous state, \textit{A\textsuperscript{y}} produces several dominant pleiotropic effects, including an increase in weight gain and body length, a susceptibility to hepatic, pulmonary and mammary tumors, and a suppression of the agouti phenotype, which results in a yellow coat color. To investigate the cellular action of \textit{A\textsuperscript{y}} with regard to its effects upon embryonic viability and adult-onset obesity, we generated a series of aggregation chimeras using embryos that differ in their agouti locus genotype. Embryos derived from \textit{A\textsubscript{y}/\textit{a}}\times\textit{\textit{A}/\textit{A}} matings were aggregated with those derived from \textit{\textit{A}/\textit{A}}\times\textit{\textit{A}/\textit{A}} matings, and genotypic identification of the resultant chimeras was accomplished using a molecular probe at the \textit{Emv-15} locus that distinguishes among the three different alleles, \textit{A\textsuperscript{y}}, \textit{A}, and \textit{a}. Among 50 chimeras, 25 analyzed as liveborns and 25 as 9.5 day embryos, 29 were \textit{\textit{A}/\textit{a}}\textsuperscript{\textasteriskcentered}\textsuperscript{\textasteriskcentered}+\textit{\textit{A}/\textit{A}} and 21 were \textit{\textit{A}/\textit{a}}\textsuperscript{\textasteriskcentered}\textsuperscript{\textasteriskcentered}→\textit{\textit{A}/\textit{A}}. The absence of \textit{\textit{A}/\textit{A}}\textsuperscript{\textasteriskcentered}+\textit{\textit{A}/\textit{A}} chimeras demonstrates that \textit{A\textsuperscript{y}/\textit{A}} cells cannot be rescued in a chimeric environment, and the relative deficiency of \textit{\textit{A}/\textit{a}}\textsuperscript{\textasteriskcentered}\textsuperscript{\textasteriskcentered}+\textit{\textit{A}/\textit{A}} chimeras suggests that, under certain conditions, \textit{A\textsuperscript{y}} heterozygosity may partially affect cell viability or proliferation. In the 25 liveborn chimeras, \textit{\textit{A}/\textit{a}}\textsuperscript{\textasteriskcentered}+\textit{\textit{A}/\textit{A}} animals became obese as adults and \textit{\textit{a}}\textsuperscript{\textasteriskcentered}\textsuperscript{\textasteriskcentered}→\textit{\textit{A}/\textit{A}} animals did not. There was no correlation between genotypic proportions and rate of weight gain, which shows that, with regard to its effects on weight gain, \textit{A\textsuperscript{y}} heterozygosity is cell non-autonomous.

Key words: agouti locus, chimera, mouse, obesity.

Introduction

Mutations that interfere with embryonic viability are important tools for understanding developmental mechanisms. In mammals, however, mutagenesis is relatively inefficient and large numbers of embryos cannot be screened expeditiously. Instead, the assumption is often made that mutations that affect different types of tissue identify genes that control fundamental aspects of embryonic development. Consequently, much attention has been focused on existing recessive lethal mutations which also exhibit pleiotropism.

The lethal yellow allele (\textit{A\textsuperscript{y}}) at the mouse agouti locus, one of the longest recognized and most intensively studied recessive lethal mutations (Castle and Little, 1910; Cuénot, 1908; Silvers, 1979), is associated with a multitude of pleiotropic effects. In the heterozygous state, \textit{A\textsuperscript{y}} alters the normal back and forth switch between eumelanin and phaeomelanin granule formation by follicular melanocytes so that only phaeomelanin granules are formed, and mice that carry \textit{A\textsuperscript{y}} (with wild-type alleles at the albino and brown loci) are entirely yellow. In addition, heterozygosity for \textit{A\textsuperscript{y}} produces increased somatic growth and obesity (Castle, 1941; Danforth, 1927), a predisposition to spontaneous and chemically induced mammary gland, pulmonary, hepatic and skin tumors (Deringer, 1970; Heston, 1942; Heston and Deringer, 1947; Heston and Vlahakis, 1961; Vlahakis and Heston, 1963), premature infertility (Granholm and Brock, 1981; Granholm et al. 1986) and glucose intolerance (Carpenter and Mayer, 1958; Hellerström and Hellman, 1963). In cases where tested, the latter effects, like those of \textit{A\textsuperscript{y}} on coat color, are dominant over the effects of all other agouti locus alleles (Silvers, 1979).

Embryos homozygous for \textit{A\textsuperscript{y}} do not survive much beyond implantation, but the exact time and site of action of \textit{A\textsuperscript{y}}-mediated recessive lethality is controversial. A multitude of morphologic abnormalities have been described in embryos derived from matings between \textit{A\textsuperscript{y}} heterozygotes (Brock and Granholm, 1979; Calarco and Pederson, 1976; Cizadlo and Granholm, 1978a; Cizadlo and Granholm, 1978b; Eaton and Green, 1962; Eaton and Green, 1963; Granholm and Johnson, 1978; Pedersen, 1974), but without an independent marker, genotypic identification of \textit{A\textsuperscript{y}/A\textsuperscript{y}} homozygotes has always been presumptive. In addition, only one study has formally tested if abnormalities in a particular cell lineage or tissue are responsible for subsequent embryonic death. Papaioannou and
Gardner (1979) performed a numerical analysis of reciprocal blastocyst injection experiments in which either the inner cell mass component or the host blastocyst component was expected to contain $A^y/A^y$ homozygotes 25% of the time. The results suggested that an $A^y/A^y$ inner cell mass could be rescued by a control blastocyst but not vice versa, pointing to a cell-autonomous defect of the trophectoderm.

These and similar experiments with recessive lethal mutations have been complicated by an inability to distinguish cells derived from homozygous embryos. To overcome these difficulties and to understand better the role of recessive lethal agouti mutations in normal development, we have used molecular markers that identify different agouti alleles to analyze aggregation chimeras between embryos of different agouti genotypes. Restriction fragment length polymorphisms (RFLPs) at the Emv-15 locus, closely linked to agouti (Copeland et al. 1983; Lovett et al. 1987; Siracusa et al. 1987b), distinguish between cells that contain zero, one or two doses of the $A^y$ mutation and have allowed us to compare the genotypic proportions in each chimera with two components of the pleiotropic phenotype — embryonic viability and increased weight gain. We now report that recessive lethal effects of $A^y/A^y$ homozygosity cannot be rescued in a chimeric environment and that adult-onset obesity in $A^y$ heterozygotes is a non cell-autonomous process.

Materials and methods

Mouse strains and mutations

The strains, C57BL/6J-/-/r and BALB/cJ, of agouti genotypes $A^y/a$ and $A/A$, respectively, were obtained from The Jackson Laboratory, Bar Harbor, Maine. Random bred CD-1 females (Charles River Laboratories) were used as uterine foster mothers.

Embryo manipulation and production of aggregation chimeras

After superovulation, embryos from $A^y/a\times A^y/a$ crosses were harvested at 1.5 days post coitum at the 2- to 4-cell stage and placed in microdrop culture for 24 h, after which most embryos had undergone a single round of division. 4- to 8-cell-stage embryos from $A^y/A^y\times A^y/A^y$ crosses were then harvested at 2.5 days post coitum and aggregated with stage-matched embryos from the $A^y/a\times A^y/a$ cross. (This protocol allowed the most efficient generation of embryos matched for cleavage stage, due to interstrain variability in the rate of in vitro preimplantation development.) Embryo recovery, dissociation, aggregation and culture were all as described previously (Cox et al. 1984).

Identification of chimera genotypes and determination of genotypic proportions

Insertion of the Emv-15 provirus is very closely associated with $A^y$ (Siracusa et al. 1987a). Furthermore, two alternative haplotypes defined by RFLPs in genomic DNA surrounding the insertion site are closely associated with the $a$ or $A$ alleles (Lovett et al. 1987; Siracusa et al. 1987b). A probe immediately 5' to the genomic insertion site of Emv-15, P0.5 (Fig. 1A), can therefore distinguish between all three alleles, $A^y$, $a$, or $A$, after digestion with HindIII. This probe detects a 2.6 kb HindIII fragment from the $A$ allele, a 3.8 kb HindIII fragment from the $A^y$ allele, and a 5.4 kb HindIII from the $a$ allele. Therefore, $A^y/A^y\leftrightarrow A/A$ chimeras will contain the 2.6 kb and 3.8 kb fragments, $a/a\leftrightarrow A/A$ chimeras will contain the 2.6 kb and 5.4 kb fragments, and $A^y/a\leftrightarrow A/A$ chimeras will contain all three fragments, 2.6 kb, 3.8 kb, and 5.4 kb, with the latter two present in equal abundance. The genetic distance between Emv-15 and $A^y$ is <0.2 cM (upper 95% confidence limit; Siracusa et al. 1987a), and the genetic distance between Emv-15 and $a$ is 0.6±0.4 cM (Siracusa et al. 1989). Therefore, although there is some question whether the $A^y$ mutation is located at precisely the same genetic map position as $A$ and $a$ (Siracusa et al. 1987a; Wallace, 1965), the likelihood of incorrectly identifying a particular agouti allele due to recombination between the agouti locus and Emv-15 is extremely small.

DNA from embryos or from tail, brain, liver and kidney samples of liveborn animals was digested with HindIII, subjected to electrophoresis through 1% agarose, and transferred to a nylon membrane by capillary action. The P0.5 probe was radiolabeled by random priming and hybridization in the presence of 10% dextran sulphate was performed according to standard procedures. For the 9.5 day embryos, 50% of the entire sample was used per lane. After an 8 day exposure, 55 of 69 embryos produced a detectable signal, but in 29 cases, chimeric genotype could not be established unequivocally (see RESULTS), because of a low recovery of DNA. For the samples from liveborn chimeras, 5-10 micrograms of DNA was used per lane. Chimeric genotypes were readily apparent in every case after a 1-2 day autoradiographic exposure, and densitometric measurement of each allele was used to estimate directly zygotic proportions.

Results

Generation and identification of adult chimeras

Animals heterozygous for the lethal yellow and non-agouti alleles were intercrossed to produce a population expected to contain 25% $a/a$ homozygotes, 50% $A^y/a$ heterozygotes, and 25% $A^y/A^y$ homozygotes. Individual embryos from this group were aggregated with developmentally matched BALB/cJ ($A/A$) embryos. In the first set of experiments, 25 liveborn animals were obtained from 89 chimeric embryos transferred into pseudopregnant mothers. Genomic DNA was recovered from a segment of each animal's tail, and a Southen blot was prepared after digestion with HindIII. Hybridization with the P0.5 probe produced the pattern of fragments expected for $A^y/a\leftrightarrow A/A$ chimeras in 11 animals and the pattern of fragments expected for $a/a\leftrightarrow A/A$ chimeras in 14 animals (Table 1). No animals produced the pattern expected for $A^y/A^y\leftrightarrow A/A$ chimeras ($X^2=16.2$, $P=0.003$ for difference from a 1:2:1 distribution).

We compared the coat color of each animal to its genotypic composition as determined by analysis of the Emv-15 RFLPs. Because the C57BL/6J-/-/r and BALB/cJ strain carry different albino alleles (C57BL/6J-/-/r is C/C and BALB/cJ is c/c), as well as different agouti locus alleles, it was necessary to consider the cellular action of both genes to interpret properly the resultant coat color patterns. The agouti locus acts
Fig. 1. Generation and identification of lethal yellow and non-agouti chimeras. (A) Breeding strategy, possible outcomes and map of the Emv-15 locus as described in the text. The asterisk indicates a polymorphic HindIII site present on chromosomes that carry the $A^Y$ and $A$ alleles, but not present on chromosomes that carry the $a$ allele. (B) An autoradiogram of tail DNA from a series of liveborn chimeras after digestion with HindIII and hybridization to the P0.5 probe. The first two lanes are $A/A$ and $a/a$ control samples as indicated. The 3.8kb fragment, seen exclusively in DNA from the $A^Y$ allele, contains approximately 450bp of cellular DNA joined to 3.35 kb of proviral DNA from Emv-15. DNA from the $A$ and $a$ alleles does not contain Emv-15, and the 2.6 kb and 5.4 kb fragments result from the presence or absence, respectively, of a polymorphic HindIII site. (C) Appearance of two chimeric mice. Coat color patches correspond to melanocyte rather than hair follicle clones.
within cells of the dermal papilla to influence the behavior of overlying follicular melanocytes, but a minimum level of tyrosinase activity, controlled by the c locus, is required to generate phaeomelanin or eumelanin (Silvers, 1961). In addition, the patch size of melanocyte clones is larger than the patch size of hair follicle clones. Because the albino (c) mutation abolishes tyrosinase activity, we expected that any patch of melanocytes that had originated from a BALB/cJ (A c/A c) progenitor would appear albino, regardless of the agouti locus genotype (-C/-C) of the underlying dermal papillae cells. Likewise, some hair follicles that contained C57BL/6J-/-v melanocytes (-C/-C) would be expected to produce agouti hairs if the underlying dermal papilla had originated from a BALB/cJ (A c/A c) progenitor.

In accord with these predictions, the observed coat color patterns included patches that were phenotypically albino, yellow or non-agouti, although, as expected, the latter two types were never seen in a single animal (Fig. 1C). In contrast, there were no large patches of agouti. However, close inspection revealed frequent but scattered agouti hairs in most animals. Animals that contained any yellow patches were scored as A y/a+/-A/A, and animals that contained any non-agouti patches were scored as a/a+/-A/A. Six animals were entirely albino and therefore could not be identified on the basis of coat color alone, but in the remainder, genotypic identification inferred from Emv-15 RFLPs. Each embryo and attached yolk sac were dissected free of maternal decidua, but, because we wished to recover as much DNA as possible, no attempt was made to separate embryonic and extraembryonic components. In the 69 embryos recovered after 157 transfers, there was a spectrum of developmental stages from early neural fold with 5-10 visible somites to early limb bud with 20-30 somites. Enough DNA was recovered to generate a hybridization signal in 54 cases, but in 29 of these the only visible DNA fragment was 2.6 kb, indicative of the A y/A y genotype. Of the remaining 25 embryos, 10 were A y/a+/-A/A or A y/a and 15 were a/a+/-A/A or a/a (Table 1).

**Distribution of chimeric genotypes**

As with the adult animals, none of the 9.5 day embryos generated the pattern expected for A y/A y+/-A/A chimeras. Furthermore, in both the adults and the embryos, the observed ratio of A y/a+/-A/A to a/a+/-A/A chimeras was distorted relative to the Mendelian expectation of 2:1 (P=0.0001 assuming non-viability of A y/A y+/-A/A chimeras) (Table 1). Because the amounts of embryo DNA are limiting, we estimate that the minimum genotypic contribution that can be detected in the 9.5 day embryos is 20%. Therefore, the existence of three groups of embryos with an apparently single genotypic contribution (1 A y/a, 2 a/a, and 29 A/A embryos) can be explained in two ways: either they represent aggregates that failed to form chimera at the morula stage, or they are actually chimerae in which the contribution of one of the genotypes is too small for detection. Both explanations could contribute to the altered ratio of A y/a+/-A/A to a/a+/-A/A.

To address the possibility that A y/a cells, in chimeric combinations with A/A, might exhibit a generalized disadvantage relative to a/a cells, we examined the frequency distribution of genotypic proportions in tail tissue from the two types of adult chimeras. Measuring the relative intensities of the 2.6 kb, 3.8 kb, and 5.4 kb HindIII fragments allowed us to estimate the proportion of A y/a or a/a cells in the tail of each A y/a+/-A/A or a/a+/-A/A chimera. (In the samples from the adult chimeras, enough DNA could be applied to a single well to detect a chimeric contribution of 5%–10%, but due to limiting amounts of DNA, a similar analysis could not be applied to the 9.5 day embryo chimeras.) Although small differences would not have been detected without applying more sensitive techniques to a larger population of animals, the frequency distributions of the proportions of A y/a or a/a cells did not show gross differences between the two types of chimeras (Fig. 2). Although these results do not exclude the possibility that A y/a cells are less effective than a/a cells in contributing to the inner cell mass following embryo aggregation, they do suggest that any possible disadvan-

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**Table 1. Generation of lethal yellow and non-agouti chimeras**

<table>
<thead>
<tr>
<th>Chimera genotype</th>
<th>No. of aggregates transferreda</th>
<th>No. recovered</th>
<th>No. genotyped</th>
<th>Experiment I.</th>
<th>Experiment II.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A y/a+/-A/A</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>89</td>
<td>157</td>
</tr>
<tr>
<td>A y/a</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>25</td>
<td>69</td>
</tr>
<tr>
<td>a/a+/-A/A</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>25</td>
<td>54b</td>
</tr>
</tbody>
</table>

*Excluding transfers to females that failed to become pregnant.
^Fractone of the embryos failed to produce a detectable hybridization signal, but, as a group, did not exhibit any readily apparent morphologic differences from the remainder of the 9.5 day embryos.

Generation and identification of 9.5 day embryo chimeras

Failure to obtain the A y/A y+/-A/A genotype in any of the 25 adult animals suggested that this class of chimeras was not surviving to term. To characterize further the developmental potential of A y/A y cells, we generated a second set of chimeras for analysis midway through gestation. As before, individual 4- to 8-cell embryos from an intercross between A y/a heterozygotes were aggregated with A/A embryos at a similar stage. Chimeric embryos were recovered at 9.5 days gestation, and constituent genotypes were determined by analysis of the Emv-15 RFLPs. Each embryo and attached yolk sac were dissected free of maternal decidua, but, because we wished to recover as much DNA as possible, no attempt was made to separate embryonic and extraembryonic components. In the 69 embryos recovered after 157 transfers, there was a spectrum of developmental stages from early neural fold with 5-10 visible somites to early limb bud with 20-30 somites. Enough DNA was recovered to generate a hybridization signal in 54 cases, but in 29 of these the only visible DNA fragment was 2.6 kb, indicative of the A y/A y genotype. Of the remaining 25 embryos, 10 were A y/a+/-A/A or A y/a and 15 were a/a+/-A/A or a/a (Table 1).
Fig. 2. Genotypic proportions in a/a→A/A and A'/a→A'/A A chimeras. In the 25 liveborn chimeras, the proportion of a/a or A'/a cells in a tail sample was estimated by comparing the densitometric signal(s) from the 5.4 kb fragment or the 5.4 kb and 3.8 kb fragments to the signal from the 2.6 kb fragment. The minimum genotypic contribution detectable in these experiments is 5%, which represents 0.5 micrograms of genomic DNA. Each symbol represents a single measurement from one animal.

Fig. 3. Growth curves of a/a→A/A and A'/a→A'/A chimeras. Animals were fed a standard diet with a fat content of 6.5% and were weighed weekly. Each curve represents a single animal.

Fig. 4. Rates of weight gain of a/a→A/A and A'/a→A'/A chimeras. The plotted value represents a linear regression of weight vs age between 10 and 20 weeks. Each symbol represents a single animal.

Adult-onset obesity in liveborn chimeras
Increased somatic growth and adult onset obesity are two of the most visible pleiotropic effects associated with the A' allele. A weight difference between yellow and non-yellow littermates first becomes apparent at 4–6 weeks of age and increases, more so in females than males, over the next 6–12 months. Central to understanding the underlying physiologic mechanism is whether increased lipid deposition in A'/a adipocytes is caused by expression of the A' gene within the adipocytes or within the environment in which they reside, i.e. whether A'-mediated obesity is cell-autonomous. The populations of a/a→A/A and A'/a→A'/A animals with different degrees of chimerism provided the opportunity to investigate this question. We expected that, if A'-mediated obesity was cell-autonomous, (1) A'/a→A'/A animals with a low proportion of A'/a cells would exhibit a weight gain similar to a/a→A/A animals; (2) A'/a→A'/A animals with a high proportion of A'/a cells would exhibit a rate of weight gain similar to A'/a animals; and (3) the proportion of A'/a cells in A'/a→A'/A chimeras would be related linearly to rate of weight gain.

Growth records indicated that, by 6 weeks of age, the range of weights was significantly different between the two types of adult chimeras (20.2±1.36 g for a/a→A/A animals; 26.2±2.75 g for A'/a→A'/A animals; P=0.0001). This difference became increasingly apparent over the next 12–14 weeks (Fig. 3). We calculated the rate of weight gain in each animal as a linear regression between 10 and 20 weeks of age and compared the frequency distributions of the two types of chimeras (Fig. 4). Rates of weight gain were similar between female and male A'/a→A'/A chimeras, but the difference relative to a/a→A/A chimeras was greater in females than in males. Although the proportion of A'/a cells in tails from A'/a→A'/A animals varied from 20% to 90% (Fig. 2), the distribution of rates of weight gain did not show a similar degree of variation. Instead, the distributions in both types of chimeras exhibited a similar degree of variation, which suggested that rates of weight gain in A'/a→A'/A animals with a low proportion of A'/a cells were indistinguishable from those with a high proportion of A'/a cells.

To address this question more directly, we compared genotypic proportion to rate of weight gain in both types of chimeras. Although we could not directly
systematically from those in solid organs, a minimal

Assuming that genotypic proportions in fat do not vary

displaced along the abscissa by 2-5 percentile units in cases

brain, kidney, or liver DNA from an adult chimera,
symbol represents a single measurement from tissue, tail,

studying the relationship of cellular genotype to a

Genetic mosaics and chimeras are valuable tools for

measure genotypic proportions in the isolated adipose
tissue compartment, we examined liver, kidney and

DNA from most of the chimeric animals in

addition to the tail DNA sampled previously. We found

no correlation between genotypic proportion and rate

of weight gain for either A\textsuperscript{y}/a\leftrightarrow A/A or a/a\leftrightarrow A/A chimeras, regardless of the tissue examined (Fig. 5).

Assuming that genotypic proportions in fat do not vary

systematically from those in solid organs, a minimal

proportion of A\textsuperscript{y}/a cells in A\textsuperscript{y}/a\leftrightarrow A/A animals is

sufficient to produce a maximal effect on rate of weight

gain, and therefore, adult-onset obesity associated with

A\textsuperscript{y} appears to be mediated by a cell non-autonomous

mechanism.

Discussion

Genetic mosaics and chimeras are valuable tools for

studying the relationship of cellular genotype to a

particular developmental phenotype. In the mouse, the
timing of X inactivation, primary sex determination, and the regulation of the switch from alphafetoprotein to albumin are most directly observed in the whole

animal, and in each case the analysis of aggregation or

blastocyst injection chimeras has played a central role in

understanding the underlying physiologic mechanisms

(Burgoyne et al. 1988; Gardner et al. 1985; Vogt et al.

1987).

We have applied the analysis of aggregation chimeras
to the study of the pleiotropic effects of the lethal

yellow allele. In 25 liveborn chimeras, none contained a

component from A\textsuperscript{y}/A\textsuperscript{y} homozygotes, which demonstrates that with regard to fetal viability, the effects of the A\textsuperscript{y} gene cannot be rescued in a chimeric environ-

ment. Neither was A\textsuperscript{y}/A\textsuperscript{y} tissue detectable in a series of

25 embryonic chimeras, which suggests that the effects of

A\textsuperscript{y} on cell viability are manifest by 9.5 days of
gestation. Finally, measurements of weight gain and

comparison of these measurements to genotypic propor-
tions in A\textsuperscript{y}/a\leftrightarrow A/A chimeras suggests that the effects of A\textsuperscript{y} on rate of weight gain are mediated by a
cell non-autonomous mechanism.

A variety of morphologic abnormalities have been
described in embryos derived from heterozygous A\textsuperscript{y}
tercrosses, including an increased frequency of

excluded blastomeres and ultrastructural abnormalities

in cleavage stage embryos (Calarco and Pederson, 1976; Pedersen, 1974), reduced growth and development of

inner cell mass and trophectoderm cultures in vitro

(Papaoianou, 1988), decreased inner cell mass number

and delayed hatching in peri-implantation blastocysts

(Cizadlo and Granholm, 1978a; Granholm and John-

son, 1978), and reduced trophectoderm differentiation

and cell death in post-implantation blastocysts (Eaton

and Green, 1962; Eaton and Green, 1963). In one of the

few studies that directly addressed tissue specificity of

A\textsuperscript{y} homozygous lethality, inner cell mass cells derived

from A\textsuperscript{y}/a\times A\textsuperscript{y}/a matings were injected into normal

(random bred) blastocysts (Papaoianou and Gardner,

1979). The recovery of 10.5 day chimeric embryos was

not significantly different from that observed in a

control experiment in which blastocysts were derived

from A\textsuperscript{y}/a\leftrightarrow A/A matings. Although these results

suggested that A\textsuperscript{y}/A\textsuperscript{y} cells can survive to at least

midgestation in a normal tissue environment, genotypic

identification of these cells was not then possible, and

we have not detected any A\textsuperscript{y}/A\textsuperscript{y} cells in our series of

chimeras. Because A\textsuperscript{y}/A\textsuperscript{y} trophectoderm cells are

likely to have been included in A\textsuperscript{y}/a\leftrightarrow A/A aggre-
gation chimeras but not in the more defined environ-

ment of A\textsuperscript{y}/A\textsuperscript{y} trophectoderm injection chimeras,

one explanation for the absence of A\textsuperscript{y}/A\textsuperscript{y} cells in our

chimeras is that even a small number of these cells in the

trophectoderm is sufficient to prevent post-implan-
tation development. Alternatively, if A\textsuperscript{y}/A\textsuperscript{y} cells were

present but too infrequent to be detected in our 9.5 day

embryos, A\textsuperscript{y}/A\textsuperscript{y} trophectoderm injection chimeras

would have contributed to the apparently non-chimeric A/A group.

However, all of the liveborn chimeras contained detectable

components from two zygotes, and therefore we expect

that any 9.5 day A\textsuperscript{y}/A\textsuperscript{y} trophectoderm injection chimeras

would not have survived until term. At present, we cannot say whether

homozygosity for A\textsuperscript{y} in inner cell mass, trophectoderm, or both tissues is responsible for embryonic death, but an analysis of blastocyst injection chimeras using

RFLPs at the Emv-15 locus should allow this question to

be addressed definitively.

The number of chimeras that contained a component

from A\textsuperscript{y}/a embryos was approximately 50% less than

expected, given that A\textsuperscript{y} heterozygosity has no effect on

viability in standard breeding experiments. One expla-

nation is that A\textsuperscript{y} heterozygosity affects cell proliferation

or viability at specific periods of embryogenesis, and

that a proportion of chimeric embryos die due to a

developmental mismatch between the A\textsuperscript{y} and non-A\textsuperscript{y}
components of the chimera. This effect might well be
masked in a normal (non-chimeric) embryo, in which
there is no developmental mismatch and a tremendous
regulative capacity in response to cell death during
early development (Snow et al. 1981). Such an effect
may have contributed to the relative excess of appar-
etly non-chimeric $A^Y/A$ 9.5 day embryos, although, as
argued above, a putative class of $A^Y/A^y\leftrightarrow A/A$ and
$A^y/a\leftrightarrow A/A$ embryos in which the $A^Y/A^y$ or $A^y/a$
component was non-detectable is unlikely to have
survived until term. Which chimeras exhibit such a
developmental mismatch could depend on the random
contribution of $A^Y/A$ and $A^y/a$ cells to a particular pre-
or peri-implantation lineage with a small number of
founder cells. For example, in chimeras in which the
$A^y/a$ component constitutes the majority of tropho-
derm, a developmental mismatch between tropho-
derm and inner cell mass might prevent subsequent
growth and differentiation of extraembryonic tissues,
the chimera would appear non-chimeric at 9.5 days
gestation, and would not survive until term. Alterna-
tively, if the $A^Y/a$ component of the chimera is, by
chance, excluded from trophoderm, the embryo
might not be susceptible to the effects of a develop-
mental mismatch and would not manifest altered genotypic
proportions in most adult tissues. This speculative
series of mechanisms constitutes a single explanation
for both the excess of non-chimeric 9.5 day embryos
and the reduced number of $A^Y/a\leftrightarrow A/A$ chimeras
among both 9.5 day embryos and liveborn animals.
However, a larger series of chimeras along with an
extensive analysis of genotypic proportions in different
tissues will be necessary to allow these issues to be
investigated more thoroughly.

Our analysis of rates of weight gain in liveborn
chimeras shows that $A^Y/a\leftrightarrow A/A$ animals with a small
proportion of $A^Y/a$ cells in tail, liver, kidney or brain,
have growth characteristics indistinguishable from ani-
mals with a large proportion of $A^Y/a$ cells. In earlier
studies directed at understanding the mechanism of $A^Y-
mediated obesity, means other than aggregation chim-
eras have been used to alter the environment of
heterozygous $A^Y$ cells. Experiments in which parabiotic
connections were established between $A^Y/A$ and $a/a$
animals suggested that a circulating substance from the
former animal could lead to increased body length in
the latter (Wolff, 1963). More recently, reciprocal
adipose tissue transplantation experiments between
$A^Y/a$ and $A/A$ animals have suggested that fat cell size
and proliferation are a function of cellular environment
rather than cellular genotype (Meade et al. 1979). Thus,
several lines of evidence now point to a cell non-
autonomous mechanism underlying $A^Y$-induced obese-
ity, but the primary physiologic changes are still un-
known. Obesity associated with $A^y$ is not caused by a
decrease in activity or an increase in nutrient intake
(Carpenter and Mayer, 1958; Cizadlo and Granholm,
1976; Fenton and Chase, 1951; Hollifield and Parson,
1957) and is not prevented by pituitary or adrenal
ablation (Jackson et al. 1976; Plocher and Powley,
1976). Although altered thermoregulation has been
described in fat yellow mice as well as in other genetic
obesity syndromes (Cizadlo et al. 1977; Herberg and
Coleman, 1977), these changes are likely to be second-
ary effects of increased body fat.

Cell-autonomous behavior of a lethal mutation is
often taken to mean that the normal gene controls an
essential intracellular process. Conversely, rescue of a
mutant phenotype may imply that the normal gene
codes for a diffusible product. These conclusions are
valid, however, only in situations in which the mutation
being tested is amorphic or null. For example, given a
particular gene that codes for a secreted protein,
expression of a mutated coding sequence might block
secretion of that protein, leading to progressive intra-
acellular accumulation and eventual cell lysis. In this
case, the mutant cells could not be rescued and cell-
autonomous death would indicate erroneously that the
normal gene product is part of an essential intracellular
process. If, on the other hand, a different mutation
totally prevented expression of the secreted protein,
mutant cells would survive if the normal protein was
provided via a chimeric environment. In this case, cell
non-autonomy would indicate correctly that the normal
gene coded for a diffusible product. It is not known if
the effects of $A^y$ on recessive lethality are due to
inactivation, altered expression, or overexpression of a
normal gene. However, we have previously reported
that a 75 kb deletion is closely associated with the
radiation-induced non-agouti lethal allele, $d$ (Barsh
and Epstein, 1989), and $d$ is lethal in combination with
$A^Y$ (Lyon et al. 1985). To this extent, $A^Y$-mediated
recessive lethality may represent a null mutation, and
the normal agouti gene product (or at least the one
affected by $A^Y$ and $d$) may indeed be part of an essential
intracellular process. In this case, the effect of $A^Y/A^Y$ or
$d/d$ cell loss on chimeric embryo survival would
depend on the time of cell death. Prior to implantation,
extensive cell death may be compensated by the regu-
late capacities of the mammalian embryo (Snow et al.
1981), but after gastrulation, significant cell death
is likely to be incompatible with embryonic survival.
Analysis of aggregation chimeras with $d$ and other
radiation-induced recessive lethal agouti mutations is
now possible and will help to resolve these questions.

Mutations induced by radiation frequently affect
multiple transcriptional units, in which case the pleio-
tropic effects of a particular mutation are more likely
to signify the proximity of one gene to the next, rather
than a single gene that affects multiple types of tissue
(Russell, 1989). There are two agouti alleles with
pleiotropic effects other than recessive lethality, $A^y$ and
$A^{Yy}$ (viable yellow), and both arose spontaneously
(Silvers, 1979). Animals heterozygous for $A^{Yy}$ exhibit
variable expression of both yellow coat color and
obesity, and variation in expression of one phenotype is
directly correlated with variation in expression of the
other (Wolff and Pitot, 1973; Wolff et al. 1986). Further-
more, like the effect of $A^y$ on obesity and female
infertility (Granholm and Dickens, 1986), suppression of
the pheomelanin to eumelanin switch is cell non-
autonomous (Silvers, 1961), supporting the possibility
that these phenotypes are mediated by the same or overlapping transcriptional units. However, although allelomorphic series for the agouti locus exist in most mammalian orders, Mus musculus is the only species in which agouti locus mutations are also known to affect growth and viability (Searle, 1968). In addition, interaction and recombination between agouti locus alleles have suggested a complex genetic structure for the locus (Russell et al. 1963; Wallace, 1954; Wallace, 1965). It is possible, therefore, that different transcriptional units mediate one or more of the pleiotropic effects associated with A\textsuperscript{y}. Resolution of these questions will require isolation of agouti locus transcripts, a goal now clearly

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References


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