Pleiotropic effects of the mouse *lethal yellow* (*A^y*) mutation explained by deletion of a maternally expressed gene and the simultaneous production of *agouti* fusion RNAs

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SUMMARY

Heterozygosity for the mouse *lethal yellow* (*A^y*) mutation leads to obesity, increased tumor susceptibility and increased activity of the *agouti* coat color gene; homozygosity for *A^y* results in embryonic death around the time of implantation. Although these pleiotropic effects have not been separated by recombination, previous studies have suggested that the dominant and recessive effects result from distinct genetic lesions. Here we use a combination of genomic and cDNA cloning experiments to demonstrate that the *A^y* mutation is caused by a 120 kb deletion which lies centromere-proximal to the *agouti* coat color gene. The deletion removes coding but not 5' untranslated sequences for a ubiquitously expressed gene predicted to encode a protein similar in sequence to an RNA-binding protein, which we named *Merc*, for maternally expressed hnRNP C-related gene, but have renamed *Raly*, since the gene is nearly identical to one reported recently by Michaud et al. (*Gene* Dev. 7, 1203-1213, 1993). The *A^y* deletion results in the splicing of *Merc/Raly* 5' untranslated sequences to *agouti* protein-coding sequences, which suggests that ectopic expression of the normal *agouti* protein by the *A^y* fusion RNA is responsible for the pleiotropic effects associated with heterozygosity for *A^y*.

We find that *Merc/Raly* RNA is present in the unfertilized egg and is also transcribed in preimplantation embryos. Using a PCR-based assay to determine the genotype of individual embryos from an *A^y/a × A^y/a* intercross, we show that, in the absence of zygotic *Merc/Raly* expression, *A^y/A^y* embryos develop to the blastocyst stage, but do not hatch from the zona pellucida or form trophoblastic outgrowths. Injection of a *Merc/Raly* antisense oligonucleotide into non-mutant embryos blocks development prior to the blastocyst stage, and can be rescued by coinjection of a *Merc/Raly* transgene. These results suggest that maternal expression of *Merc/Raly* plays an important role in preimplantation development and that its deletion of is sufficient to explain *A^y*-associated embryonic lethality.

Key words: *agouti*, *lethal yellow*, hnRNP, mouse development

INTRODUCTION

In 1905, mice carrying the *lethal yellow* mutation (*A^y*) were used to provide the first evidence that heritable traits could be used to study vertebrate development (Cuénot, 1905). Inviability of *A^y/A^y* animals, revealed initially by a deviation from Mendelian proportions in the offspring of *A^y/^A^y × A^y/^A^y* intercrosses, has been the subject of many embryologic studies over the last several decades [reviewed in Magnuson (1986); Silvers (1979)]. Morphologic and ultrastructural abnormalities attributed to the effects of the *A^y* mutation include degeneration of trophectoderm in the peri-implantation blastocyst (Eaton and Green, 1963), an increased frequency of excluded blastomeres in cleavage-stage embryos (Calarco and Pedersen, 1976; Pedersen, 1974), an inability of embryos to form outgrowths in culture (Papaioannou, 1988) and failure to hatch from the zona pellucida when placed into diapause (Papaioannou and Gardner, 1992). Although the variety of defects observed may be due to a combination of factors including different genetic backgrounds, variable expressivity of the mutation, and the lack of an independent marker to determine the genotype of individual embryos, these studies suggest that the effects of *A^y* are most evident around the time of implantation. Because abnormalities are observed in both the TE and the inner cell mass (Papaioannou, 1988), and because *A^y/A^y* cells cannot be rescued in aggregation chimeras (Barsh et al., 1990), *A^y* may affect a critical developmental step required prior to implantation.
A\(^y\) is also one of the oldest recognized mutations of the agouti coat color locus, in which alleles associated with the synthesis of yellow pigment in hair follicles are dominant to those associated with the synthesis of black pigment [reviewed in Silvers (1979)]. A\(^y\) in combination with all other agouti locus alleles leads to the exclusive production of yellow pigment and, in addition, has pleiotropic effects, including adult onset obesity and increased somatic growth (Carpenter and Mayer, 1958; Castle, 1941; Danforth, 1927), increased susceptibility to tumor formation (Heston and Deringer, 1947; Heston and Vlahakis, 1961; Vlahakis and Heston, 1963) and premature infertility (Granholm and Dickens, 1986; Granholm et al., 1986).

The agouti gene has been cloned recently and is thought to encode a signaling molecule that directs follicular melanocytes to switch from the synthesis of black pigment, eumelanin, to yellow pigment, phaeomelanin (Bultman et al., 1992; Miller et al., 1993). For most agouti genotypes, agouti RNA is expressed only in the skin during the time of phaeomelanin synthesis. However, A\(^y\) is associated with the ubiquitous expression of a chimeric RNA that fuses a novel 5' end, 'exon 1A\(^y\)', to agouti-coding sequences at its 3' end (Miller et al., 1993). This suggests that some or all of the pleiotropic effects of A\(^y\) are due to ectopic expression of the normal agouti protein.

Three separate arguments suggest that lethality of A\(^y\)/A\(^y\) animals may not result from altered expression of the agouti coat color gene. First, the effects of A\(^y\) on coat color are a gain-of-function, yet embryonic lethality is most likely a loss-of-function (Barsh and Epstein, 1989; Barsh et al., 1990). Second, the viable yellow (A\(^y\)) mutation has similar effects to A\(^y\) with regard to obesity and tumor susceptibility, but is not lethal when homozygous (Dickie, 1962; Wolff et al., 1986). Finally, genetic complementation analysis suggests that there are multiple genes close to or within the agouti locus required for embryonic development [Barsh and Epstein, 1989; Lyon et al., 1985; Russell et al., 1963]; reviewed in Siracusa (1991)].

To understand the molecular basis for A\(^y\)/A\(^y\)-associated lethality better, we have performed a combination of molecular and embryologic studies. We find that the A\(^y\) chromosome contains a 120 kilobase (kb) deletion that lies centromere-proximal to all previously identified agouti exons. The deletion removes most of the coding sequences for a gene that is likely to encode an RNA-binding protein that we have named Merc, and results in a novel mutational mechanism in which the non-deleted first exon of the Merc gene is spliced to protein-coding sequences of agouti. Based on the developmental defects that we observe both in A\(^y\)/A\(^y\) embryos and in normal embryos in which expression of the Merc gene has been inhibited experimentally, we conclude that loss of Merc function is responsible for A\(^y\)-associated embryonic lethality.

**MATERIALS AND METHODS**

**Mouse strains and mutations**

Mice carrying the A\(^y\), A\(^w\) and A alleles are propagated in our laboratory as C57BL/6J-A\(^y\)/a, 129/SvJ-A\(^w\)/A\(^y\) and FVB/N-A\(^w\), respectively. The C57BL/6J and 129/SvJ strains were obtained from The Jackson Laboratory and the FVB/N strain was obtained from Taconic (Germantown, NY). Mice carrying the a\(^o\) mutation were generously provided by Dr Virginia Papaioannou (Tufts University School of Medicine, Boston, MA) as a balanced lethal stock A\(^o\)/a\(^o\); the a\(^o\) allele is propagated in our laboratory by backcrossing to C57BL/6J-a\(^o\) animals.

**Genomic cloning, cDNA cloning and DNA probes**

We have previously described the isolation and structure of cosmid clones that constitute a 60 kb contig containing agouti exons 1B, 1C, 2, 3 and 4 (Miller et al., 1993) (The agouti exon nomenclature used in the present work differs from that used previously; the rationale is summarized in Fig. 1). As it became apparent that alternative isoforms of the agouti cDNAs had 5' ends that were not contained within these cosmid clones, the contig was extended further 5' with regard to the direction of agouti transcription by screening a commercial bacteriophage P1 library (Genome Systems, St Louis, MO) with primer pairs derived from agouti cDNAs or genomic clones. The commercial P1 library was constructed from a mouse fibroblast cell line, C127, derived from the RIII mouse strain. The RIII strain carries the A allele, which is consistent with our observation that, in regions of overlap, the P1 clones that we obtained are identical to genomic clones derived from DNA of mice that carry the A allele.

The P1 clones P1-24 and P1-25 were isolated using oligonucleotide primers from a subclone of cosD3 (see Fig. 1), 5'-CTTAG-GTTTCTCTGTGTCCCC-3' and 5'-CAGAAGTCTCCG-GATGGTGC-3', that amplify a 225 base pair fragment containing agouti exon 1B. The P1 clones P1-74 and P1-75 were isolated using oligonucleotide primers from exon 1A, 5'-AGTCTAGTCG-CTTGAGCCTTC-3' and 5'-TGGGACCCCCGGTGTTGTC-3', that amplify a 78 base pair fragment. A P1 clone that contains the 5' end of the A\(^y\)-specific cDNA, P1-62, was isolated using the oligonucleotide primers 5'-CCGGAAGGGCGGGGACCCG-3' and 5'-CCGGCGCAGAGTCTGAGAG-3', that lie at the ends of exon 1A (see Fig. 1 and Fig. 4) and amplify a 147 base pair fragment. The BamHI restriction maps of the P1 clones were determined by partial digestion and indirect end labeling. Comparison of restriction maps allowed a 160 kb contig to be generated that included all the cosmids and P1-24, P1-25, P1-74, and P1-75 (Fig. 1). The P1-62 clone is approximately 100 kb in length but does not overlap with any of the cosmid or bacteriophage P1 clones.

A 1.0 kb BamHI-EcoRI genomic fragment that contains exon 1A was subcloned from P1-62 and is described below as the 'exon 1A probe'. A 1.3 kb Xbal-BamHI genomic fragment that contains exon 1A was subcloned from P1-75 and is described below as the 'exon 1A probe'. A 1.3 kb Xbal-BamHI fragment subcloned from one of the Merc cDNA clones contains the entire open reading frame and corresponds to residues 403-1720 in Fig. 4A; it is described below as the 'Merc cDNA probe'. In some experiments, an internal 340 bp Smal-ClaI fragment was used instead of the 1.3 kb Xbal-BamHI fragment. A 1.0 kb Xbal-EcoRI genomic fragment that contains agouti exon 4 is described below as the 'exon 4 probe' (The same probe d'). To isolate the Merc cDNA, a commercial cDNA library prepared from the teratocarcinoma cell line PCC4 (Stratagene, San Diego, CA) was screened initially with the exon 1A probe. Two clones were recovered, 1.7 kb and 0.8 kb in length; each clone contained exon1A sequences followed by an open reading frame at the 3' end, but neither clone contained a poly(A) sequence or an obvious polyadenylation signal. A 340 basepair (bp) fragment from the 3' end of the 1.7 kb clone was then used to rescreen the library, and a third clone was isolated, 1.5 kb in length, that contained the entire Merc open reading frame followed by a consensus polyadenylation sequence and a poly(A) tail.

**RNA expression studies**

Expression of the Merc RNA in adult tissues was determined by northern hybridization using the 147 bp exon 1A fragment as a hybridization probe. For PCR-based studies of embryos, total RNA extracted from approximately 50 mouse eggs, 2-cell embryos, or from
25 blastocysts as previously described (Andria et al., 1992) was reverse transcribed using an oligonucleotide primer complementary to sequences in the 3′ end of the Merc open reading frame (see Fig. 4A), 5′-ACCATCATCTGCAATTG-3′, and then PCR-amplified using the oligonucleotide primers 5′-CAGGGCCGCTCCTTC-3′ and 5′-TGTCACAGGACACCAG-3′. The same group of primers were used to analyze RNA from neonatal skin of A/WAy mice. These primers span at least one Merc intron that separates 5′ untranslated from protein-coding sequences, and produce fragments of 342 bp or 259 bp depending on the presence or absence, respectively, of an alternatively spliced 83 bp exon (see Figs 4, 5). The identity of the products was confirmed both by direct sequencing and by Southern hybridization with the Merc cDNA probe.

For embryo expression studies of the Merc/agouti fusion RNA, total RNA was extracted from approximately 50 a/a eggs, or from 25 blastocysts derived from A/A males mated to a/a females, in which fusion RNA must arise from the paternal chromosome. The RNA was reverse transcribed using an oligonucleotide primer, 5′-GAG-GGAAATCTACCAGGCCATCTTCATCGA-3′, complementary to sequences within the agouti protein-coding region, and then PCR-amplified using the oligonucleotide primers 5′-CAGGGCCGCCTCCTTC-3′ and 5′-CGAGTTCTAGGAGGATCTCCGGC-3′. These primers are located in Merc 5′ untranslated and 3′ protein-coding sequence, respectively, and amplify a 386 bp or 229 bp fragment depending on the presence or absence of agouti exons 1A and 1A′ (see Fig. 5). The identity of the products has been confirmed by hybridization with an internal oligonucleotide probe from agouti exon 2, 5′-CGAGGAAATCTACCATGAGTGTCACCCCCCCTACTC-3′.

Embryologic studies

To determine the developmental potential of A/A′ embryos, C57BL/6J-A/y animals were intercrossed in natural matings, blastocysts were recovered by uterine flushing at 3.5 days post coitum (dpc), and placed into vitro microdrop culture with DMEM supplemented with 10% fetal calf serum. After 3–4 days, the development of each embryo was assessed with regard to hatching from the zona pellucida, attachment and trophoblastic outgrowth. After phenotypic scoring, the agouti locus genotype of each embryo was determined using a PCR-based assay as previously described (Frohman et al., 1993). The assay determines inheritance of a variant HindIII site, present on the A′ allele and absent from the a allele, located ±0.2 cM distal to A′ (Siracusa et al., 1987).

To determine whether inhibition of Merc gene expression affected development of non-mutant embryos, a sense or antisense oligodeoxynucleotide that spanned the predicted Merc translational initiation site was injected into fertilized 1-cell embryos obtained from superovulated FVB/N or C57BL/6J mice. The oligodeoxynucleotides, 5′-TTCAGGACATTGGTCTCAC-3′ (antisense) and 5′-GTGAAA-CACCATGTCCCGA-3′ (sense), were synthesized with phosphorothioate linkages at the first and last 5′ residues to decrease susceptibility to intracellular degradation. An additional control ‘scrambled’ oligodeoxynucleotide with a base composition similar to the antisense oligodeoxynucleotide, a Merc or control transgene was coinjected with oligonucleotide at a concentration of 1 μg/ml, which corresponds approximately to 10,000 molecules of 2 kb fragment injected per embryo. The Merc transgene was constructed by fusing the PGK promoter from the vector pNT (Tybulewicz et al., 1991) to a 1.3 kb Xhol–EcoRI fragment that contained the coding sequences of the Merc gene (residues 403–1726 of the Merc cDNA; see Fig. 4); the transgene was then excised as a 1.9 kb EcoRI fragment. A 2 kb control transgene was also used that contained agouti-coding sequences. In a first series of experiments, the embryos were injected with undiluted sense or antisense oligodeoxynucleotide, transferred into pseudopregnant foster mothers for 3 days, and then placed into microdrop culture as described above. In subsequent experiments, development of cleavage stage embryos was assessed by transferring injected embryos into microdrop culture and scoring their phenotype with regard to cell number and/or cell lysis on subsequent days.

Additional techniques

High molecular weight DNA for pulsed field gel electrophoresis was prepared from homogenized spleen tissue and digested with ‘ rare-cutting’ restriction enzymes as previously described. Contour clamped homogenous electric field (CHEF) electrophoresis was performed using a commercial apparatus (CBS Scientific, San Diego, CA) at 150 V, in 0.5× TBE (45 mM Tris, pH 8.4, 45 mM boric acid, 1 mM EDTA) and 0.1 μg/ml ethidium bromide, at 6°C. Direction of the electric field was changed according to a ramped program from 40 to 140 sec, using 1 second intervals, and total electrophoresis time was ≈48 hours. Molecular weight estimations were based on a yeast subline derived from strain AB1380, which carries 245 kb, 290 kb, 370 kb, 460 kb, 580 kb, 630 kb, 700 kb, 770 kb, 800 kb, 850 kb and 945 kb chromosomes. These maps differ by virtue of (1) a 120 kb deletion present only in the A′ allele which is described below; and (2) an approximately 20 kb insertion present only in the a allele between agouti exons 1C and 2 (Bultman et al., 1992; unpublished observations).

Northern hybridizations, Southern hybridizations and DNA sequence analysis using modified T7 polymerase and dideoxy chain termination were performed according to standard techniques (Sambrook et al., 1989) using radiolabeled nucleotides and hybridization in the presence of 10% dextran sulfate. Reverse transcription and PCR protocols are described in Miller et al. (1993).

RESULTS

Identification of an A′-specific first exon of agouti

In the course of cloning cDNAs from A/A′ mice, a chimeric RNA was identified in which a 147 bp A′-specific sequence (exon 1Ay) was spliced to other exons that contained agouti-coding sequences (Miller et al., 1993). The analysis of these chimeric RNAs was complicated by the normally heterogeneous nature of the 5′ end of agouti mRNA, in which we have found three different first exons (1A, 1B, 1C) and one alternatively spliced exon (1Ay) in cDNAs from A/A and A*/A′ mice (Fig. 1). Of 17 cDNA clones isolated from A/A′ skin RNA, exon 1Ay was fused to agouti exon 1A in six clones, and exon 1Ay was fused to agouti exon 2 in four clones. The remaining seven cDNA clones did not contain exon 1Ay, but instead began with agouti exons 1B or 1C. Fusion of exon 1Ay to agouti exon 2 uses the same splice acceptor used by agouti exons 1A, 1A′, 1B, and 1C. However, fusion of exon 1Ay to agouti exon 1A appears to use a cryptic splice acceptor, 5′-
ccaacatgac|GAA-3′, since this entire sequence is present normally in exon 1A (see below, Fig. 4).

The A' chromosome contains a 120 kb deletion between exon 1Ay and agouti exon 1A

To investigate if a chromosomal or subvisible rearrangement caused the A' mutation, we first determined whether the normal chromosomal location of exon 1Ay lay close to the agouti gene on the distal portion of mouse chromosome 2. Oligonucleotide primers at the ends of exon 1Ay were used to amplify DNA from laboratory mice, a hamster cell line (GM 459), and a mouse/hamster hybrid cell line that carries most of mouse chromosome 2 (ADCT-25). DNA was also amplified from 4 irradiation-reduced somatic cell hybrids which have been shown previously to retain mouse DNA markers that lie within approximately 5 cM of the agouti gene (Ollmann et al., 1992). A PCR product specific for exon 1Ay was observed with the laboratory mouse DNA, ADCT-25 DNA, and one radiation hybrid DNA, RH33, but not with GM 459 parent hamster DNA (Fig. 1C). These results indicate that exon 1Ay is derived from a region of mouse chromosome 2 that lies close to the normal agouti gene.

Hybridization of exon 1Ay to cloned genomic DNA indicated that exon 1Ay was not located within a 160 kb cosmid and bacteriophage P1 contig (Fig. 1A) that contains the entire 125 kb agouti gene (data not shown). In addition, a 100 kb mouse genomic P1 clone that contains exon 1Ay did not overlap with the agouti contig, suggesting that exon 1Ay was located at least 150 kb away from agouti exon 1A. To determine more precisely the physical relationship between exon 1Ay and the agouti gene, we used genomic probes and pulsed field gel electrophoresis to construct a long-range restriction map of both the A' and non-mutant chromosomes.

DNA samples from animals of agouti genotypes A'A, A'a', a'a, or a'/? were digested with BssHII, EagI, or Smal, fractionated on an agarose gel in the 50-750 kb size range, transferred to a nylon filter, and hybridized separately with genomic probes that contained exon 1Ay or agouti exons (Fig. 2). The results obtained with a probe that contained agouti exon 1A were identical to those obtained with a probe that contained agouti exon 4 (data not shown). For some enzyme/probe combinations, two fragments were observed with DNA from at
least one heterozygote but not from *a/*a homozygotes, and comparison of the results allowed us to identify the allelic origin of each fragment (Table 1, Fig. 2). The exon 1Ay and *agouti* exon 1A probes detect the same *a*-specific 320 kb and *Ay*-specific 180 kb *BssHII* fragments. Similarly, these probes detect the same *a*-specific 345 kb and *Ay*-specific 205 kb *SmaI* fragments. These results indicate that exon 1Ay and *agouti* exon 1A are located a maximum of 320 kb apart in the *a* chromosome and a maximum

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**Table 1. High-molecular-weight restriction fragments detected by probes that span the *Ay* deletion**

<table>
<thead>
<tr>
<th>probe</th>
<th>BssHII</th>
<th>EagI</th>
<th>SmaI</th>
</tr>
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<tbody>
<tr>
<td>exon 1Ay</td>
<td>320</td>
<td>180</td>
<td>180†</td>
</tr>
<tr>
<td><em>Merc</em> cDNA</td>
<td>320</td>
<td>–</td>
<td>180</td>
</tr>
<tr>
<td>exon 1A; exon 4</td>
<td>320</td>
<td>180</td>
<td>220</td>
</tr>
</tbody>
</table>

*Estimated sizes are in kilobases based upon the results shown in Figure 2, and are based on the mobility of yeast chromosomes in adjacent lanes. Probes are described fully in the Materials and Methods and in Figure 2. Comparison of the fragments observed in the *a/*a and *Ay/*a DNA samples allowed us to determine their chromosomal origin as "*a*" or "*Ay*" with one exception as noted below.

†The exon 1Ay probe detects a single *EagI* fragment of 180 kb in *A/*a DNA. The results obtained with *BssHII* and *SmaI* indicate that the probe is present in both alleles.

‡Absence of the 180 kb *EagI* fragment from the *Ay* allele is based on the results obtained with *BssHII* and *SmaI*. 
of 180 kb apart in the A\(^{y}\) chromosome. Furthermore, the difference between the α-specific and A\(^{y}\)-specific fragments is the same for BssHII and SmaI, suggesting that a deletion of 120 kb has occurred in the A\(^{y}\) chromosome. (The deletion is 20 kb less than the difference between the two fragments, 140 kb, due to a 20 kb insertion in the α chromosome that is not present in the A\(^{y}\) chromosome). Similar results were obtained for the enzyme combinations MluI+Sall and NotI+Sall (data not shown).

Using a combination of Southern hybridization and DNA sequence analysis, positions have been determined for the BssHII, EagI, and SmaI sites that are susceptible to cleavage in genomic DNA. The BssHII and EagI sites are located at the 3′ end of agouti exon 4, and the SmaI site is located 25 kb further 3′ (Fig. 2). Applying this information to construct a long-range restriction map indicates that the 120 kb A\(^{y}\) deletion removes a EagI site that lies 200 kb 5′ to agouti exon 4, and that normally separates exon 1Ay and agouti exon 1A (Fig. 2).

Fig. 4. Sequence and splicing pattern of the Merc gene. (A) Composite sequence obtained from three overlapping cDNAs isolated from a teratocarcinoma cell line. The 147 bp exon 1Ay sequence and a consensus polyadenylation signal have a single underline, and the alternatively spliced 83 bp exon has a double underline. The predicted protein sequence as translated from the first methionine codon is shown from residues 483-1370. (B) Diagram of alternative splicing and exon usage in the A\(^{y}\) and non-mutant chromosome as determined by PCR-based studies. The A\(^{y}\)-specific pattern of splicing was determined by comparison of the composite Merc cDNA sequence to the sequences of cDNA clones shown in Fig. 1. A\(^{y}\)-specific cDNAs that contain agouti exon 1A do not contain its entire 5′ end, suggesting that inclusion of exon 1A in Merc/agouti fusion RNAs is due to a cryptic splice acceptor. The non-mutant pattern of splicing was determined by using oligonucleotide primers in 5′ untranslated and potential Merc-coding regions to PCR-amplify cDNA from neonatal A\(^{W}\)/A\(^{W}\) skin RNA as described in Materials and Methods. Direct sequencing of the gel-purified products (see text) revealed two patterns of splicing that differ in their inclusion of an 83 bp region. The genomic sequence following Merc exon 1 is identical to the first 4 nucleotides of this 83 bp region, referred to as Merc exon 1′ in the figure, and contains two potential splice donor sites (see text). (C) Alignment of potential Merc protein-coding sequences with those of the human hnRNP C protein. The RNA-binding domain in hnRNP C consists of a four-stranded beta-pleated sheet with two alpha helices whose positions are indicated.
Exon 1Ay represents the 5’ end of a ubiquitously expressed cDNA that is deleted from the A’ chromosome

A simple model to explain both the 120 kb deletion and the chimeric exon 1Ay-agouti mRNA associated with the A’ mutation postulates that the deletion removes the 3’ portion of a gene that begins with exon 1Ay, and juxtaposes the first intron of this gene with 5’ flanking sequences of agouti exon 1A. To determine if exon 1Ay might be contained normally in a gene other than the A’-specific chimeric mRNA, we used exon 1Ay as a probe to examine RNA from animals of different agouti genotypes by northern hybridization analysis. In most tissues examined of A’ heterozygotes, the exon 1Ay probe detected two RNAs, approximately 1.0 kb and 1.7 kb in length (Fig. 3). In all tissues examined of A’/A’ animals, however, only the 1.7 kb RNA was present. Probes from the agouti cDNA detect only the 1.0 kb RNA in A’ heterozygotes, indicating that the 1.0 kb RNA represents the chimeric A’-specific RNA, and that the 1.7 kb RNA represents a different gene.

To isolate cDNAs representative of the 1.7 kb RNA, we used an exon 1Ay probe to screen a teratocarcinoma cDNA library. Two cDNA clones were identified among 6 × 10⁵ plaques screened; subsequently, an internal probe from one of these clones was used to rescreen the library and a third clone was isolated. A composite sequence from these three cDNA clones is 1760 bp long including a 35 bp region of poly(A) at the 3’ end (Fig. 4A). The sequence contains a 296 amino acid open reading frame with putative 5’ untranslated regions 482 bp and 355 bp in length, respectively. Based on the sequence and expression pattern of the cDNA (see below), we refer to it as Merc, for maternally expressed hnRNP C-related gene.

To investigate the physical relationship of the Merc cDNA to the 120 kb A’ deletion, we hybridized a cDNA fragment that contained the Merc open reading frame to nylon filters in which A’-specific large restriction fragments had been defined previously (Fig. 2). The Merc open reading frame probe did not hybridize to any A’-specific fragments, but did detect the same α-specific BstIII, EagI, and SmaI fragments as the exon 1Ay probe, indicating that most or all of the Merc open reading frame was contained in the 120 kb A’ deletion (Fig. 2).

The Merc cDNA sequence is alternatively spliced and predicts an RNA-binding protein

The entire exon 1Ay sequence, 147 bp in length, is colinear with both the Merc cDNA (residues 244–390) and the corresponding genomic sequence. Surprisingly, the Merc cDNA and the genomic sequence are identical for an additional 4 nucleotides beyond the end of exon 1Ay, at which point they diverge from each other (Fig. 4B). To investigate whether alternative usage of splice donors used normally by the Merc gene might account for this difference, cDNA produced from A’/A’ skin was PCR-amplified with oligonucleotide primers that spanned the interval from exon 1Ay to the Merc open reading frame, and the products were characterized by gel electrophoresis and direct sequencing. Two products were obtained in an approximate 3:1 ratio (see Fig. 5A for example), gel purified and sequenced using an internal primer. The major (>75%) product was identical to the Merc cDNA; however, the minor (<25%) product was missing an 83 bp region (described as Merc exon 1’, see Fig. 4B) that began at the end of exon 1Ay and that did not affect the Merc open reading frame. Although further genomic sequence analysis will be required to distinguish whether the major Merc cDNA product is produced by an 83 nt exon that uses the same splice donor as that used by exon 1Ay, or by a 79 nt exon that uses a different splice donor from the one used by exon 1Ay, these results indicate that the Merc gene normally produces two isoforms, at least one of which uses the same splice donor used by exon 1Ay.

The methionine codon that initiates the 296 amino acid Merc open reading frame at nucleotide residue 483 is the first ATG in the Merc cDNA sequence, and lies within a region, 5’-ACCAGTCC-3’, predicted to function as a site for eukaryotic translational initiation. Sequence similarity searches of computerized databases revealed 43% identity with the human protein heterogeneous nuclear ribonucleoprotein particle C (hnRNP C) (Swanson et al., 1987). A comparison (Fig. 4C) indicates that most of the similarity lies within a 90 residue amino terminal region (80% identity), which includes the RNA-binding domain (Gorlach et al., 1992; Wittekind et al., 1992).

Merc is normally expressed in the unfertilized egg and the blastocyst

Because the Merc open reading frame is contained within the A’-associated 120 kb deletion (see above), we considered
whether failure to express *Merc* might be responsible for *A/y*-associated embryonic lethality. Although hnRNP C and other hnRNPs are well characterized biochemically, little is known regarding their expression in development. To investigate whether *Merc* was expressed at or prior to the time at which *A/yA/y* embryos are thought to exhibit morphologic abnormalities, RNA from 25-50 eggs, 2-cell embryos, or blastocysts was reverse transcribed and PCR-amplified with the same oligonucleotide primers used to analyze *Merc* expression in skin (Fig. 4B). Gel electrophoresis and Southern hybridization of the amplified products indicated that both forms of the *Merc* RNA (with and without *Merc* exon 1, Fig. 4B) were present at relatively high levels in unfertilized eggs and relatively low levels in blastocysts (Fig. 5A). To determine whether the blastocyst RNA was likely to represent embryonic synthesis and/or degradation of maternal stores, expression of the *Merclagouti* fusion RNA was examined in embryos derived from an *A/yA/y* male crossed to an *a/a* female, in which the only source of the fusion RNA is the *A/y* chromosome provided by the male genome. The fusion RNA was detectable in *A/yA/y* blastocysts but not in *a/a* blastocysts or in *a/a* unfertilized eggs (Fig. 5B), suggesting that *Merc* RNA is expressed embryonically as well as maternally.

*A/yA/y* embryos fail to hatch in vitro

Previous observations of embryos derived from *A/y− × A/y−* intercrosses have reported a wide range of developmental defects in 25% of the animals compared to control crosses (Calarco and Pedersen, 1976; Eaton and Green, 1963; Johnson and Granholm, 1978; Papaioannou, 1988; Papaioannou and Gardner, 1992; Pedersen, 1974). However, most of these studies are complicated by the inability to determine the genotype of individual embryos. To determine if *A/yA/y* embryos exhibited morphologic or developmental abnormalities prior to implantation, 3.5 dpc blastocysts were recovered from *A/yA/y × A/yA/y* matings, placed into culture and their development was scored after 2-3 days. The genotype of each embryo was determined using a PCR-based assay to evaluate the presence of a variant *HindIII* site at the *Env-15* locus, which is located less than 0.2 cM distal to *A/y* (Siracusa et al., 1987). Of 92 embryos evaluated, 65 hatched and formed trophoblastic outgrowths, while 27 never hatched (Table 2). Based on the identity of the variant *HindIII* site, 14/27 that did not hatch were *A/yA/y*, while no *A/yA/y* embryos were found among the 65 embryos that did hatch.

**Inhibition of *Merc* expression blocks development of cleavage-stage embryos**

Although the expression pattern of the *Merc* gene is consistent with a role in *A/y*-associated embryonic lethality, the 120 kb deletion in the *A/y* chromosome may remove additional genes that contribute to the failure of *A/yA/y* embryos to develop past the blastocyst stage. To separate effects due to loss of *Merc* expression from other effects due to the *A/y*-associated deletion, we examined the development of non-mutant embryos injected with *Merc* antisense oligodeoxynucleotides.

In a first series of experiments, approximately 5×10⁶ molecules of sense or antisense oligodeoxynucleotides that spanned the predicted translational initiation site of *Merc* were injected into 1-cell fertilized embryos and the embryos were transferred into the oviducts of pseudopregnant females. At 3.5 dpc, embryos were recovered from the uterus, placed into in vitro culture and their development compared to each other and to uninjected embryos. Of 169 1-cell embryos injected with the *Merc* antisense oligodeoxynucleotide, only 76 (45%) were recovered from the uterus compared to 87% and 77% for the uninjected and sense controls, respectively (Fig. 6). Nearly all of the embryos injected with the antisense oligodeoxynucleotide appeared morphologically abnormal at 3.5 dpc; most had not developed beyond the 4-cell stage and many appeared to

![Fig. 5. Embryonic expression of the *Merc* and the *Merclagouti* fusion RNAs. (A) After reverse transcription of RNA from approximately 25-50 C57BL/6J-ala unfertilized eggs, 2-cell embryos, or blastocysts, *Merc* cDNA was PCR-amplified using oligonucleotide primers as indicated in the diagram and as described in Materials and Methods. Three fragments were evident after ethidium bromide staining or hybridization with the *Merc* cDNA probe as shown. Direct sequencing of the gel-purified products indicated that the lower fragment was the 255 bp product (which lacked the 83 bp exon shown in Fig. 4B), and that both upper fragments were different forms of the 342 bp product (which contained the 83 bp exon). (B) After reverse transcription of RNA from approximately 25-50 C57BL/6J-ala unfertilized eggs, blastocysts or *A/yA/y* blastocysts in which the *A/y* chromosome was provided by the male parent, *Merclagouti* fusion cDNA was PCR-amplified using oligonucleotide primers as indicated in the diagram and as described in Materials and Methods. The predominant fragment detected by hybridization to an internal oligonucleotide probe from *agouti* exon 2 is a 386 bp product that contains *agouti* exons 1A and 1A'.](image-url)
exhibit lysis of individual blastomeres compared to embryos injected with the sense oligonucleotide (Fig. 7). By 5.5 dpc, only 3 of the 76 embryos (4%) injected with the Merc antisense oligonucleotide and recovered from the uterus had developed into blastocysts, compared to 61% and 23% for the uninjected and sense, respectively (Fig. 6). The 3 blastocysts that did develop from embryos injected with the Merc antisense oligonucleotide were probably abnormal, since none of them hatched from the zona pellucida by 6.5 dpc, compared to 87% and 69% for the uninjected and sense controls, respectively (Fig. 6).

In a second series of experiments designed to examine the time course and concentration dependence of developmental arrest induced by injection of the Merc antisense oligonucleotide, 1-cell fertilized embryos were injected with approximately $5 \times 10^5$, $1 \times 10^7$, or $2 \times 10^7$ molecules of sense or antisense oligonucleotide, transferred immediately into microdrop culture, and their development was scored with regard to cell number and/or blastomere lysis for the next 4 days. At the two higher concentrations, an effect of the antisense compared to the sense oligonucleotide was apparent 1 day after injection—blastomere lysis was observed in 22% or 19% of the embryos injected with $5 \times 10^5$ or $1 \times 10^7$ molecules of antisense oligonucleotide, respectively, but in none of the embryos injected with the same amount of sense oligonucleotide (Fig. 8). By 4.5 dpc, an effect of the antisense compared to the sense oligonucleotide was apparent in embryos injected with all three oligonucleotide concentrations—only 2.8%, 29%, or 12% of embryos injected with $5 \times 10^5$, $1 \times 10^7$, or $2 \times 10^7$ molecules of antisense oligonucleotide, respectively, had developed into blastocysts compared to 18%, 90%, or 73%, of embryos injected with the corresponding amount of sense oligonucleotide (Fig. 8; Table 3). The effect of the antisense oligonucleotide at 4.5 dpc was also apparent when compared to embryos injected with a control ‘scrambled’ oligonucleotide (Table 3).

In a third series of experiments designed to examine whether developmental arrest induced by the Merc antisense oligonucleotide could be rescued by expression of the Merc transgene, embryos were co-injected with different amounts of antisense oligonucleotide and a Merc transgene, and their development was compared by scoring the proportion of injected embryos that had developed into blastocysts by 4.5 dpc. With $5 \times 10^8$ molecules of antisense oligonucleotide, coinjection of the Merc transgene did not significantly affect the proportion of embryos developing into blastocysts, 9.5%, when compared to antisense oligonucleotide alone, 2.8% ($P=0.24$, Table 3). However, with $1 \times 10^7$ molecules of antisense oligonucleotide, coinjection of the Merc transgene resulted in partial rescue; 53% of co-injected embryos developed into blastocysts compared to 27% injected with antisense oligonucleotide alone ($P=0.017$, Table 3). Finally, coinjection of the Merc transgene completely rescued embryos injected with $2 \times 10^5$ molecules of antisense oligonucleotide; 76% of co-injected embryos developed into blastocysts compared to 12% injected with antisense oligonucleotide alone ($P=0.0001$, Table 3).

### DISCUSSION

The $A^y$ mutation has intrigued developmental biologists for 90 years due to its pleiotropic effects on coat color, regulation of body weight, tumor susceptibility and embryonic development. Here we show that a 120 kb deletion in the $A^y$ chromosome removes potential protein-coding sequences for a maternally expressed gene, Merc, that we have isolated from a teratocarcinoma cDNA library, and which is highly similar to the human hnRNP C gene. The deletion also results in the production of a chimeric RNA in which ubiquitously expressed Merc S' untranslated sequences are fused to protein-coding sequences of the agouti gene (Fig. 9). During the preparation of this manuscript, isolation of a gene nearly identical to Merc was reported by Michaud et al. (1993). These investigators used exon 1Ay sequences to isolate a 1.4 kb cDNA from a postimplantation mouse embryo cDNA library which had the potential to encode a gene they named Raly, for RNA-binding

Table 2. Development in vitro of embryos derived from an $A^y/a \times A^y/a$ intercross

<table>
<thead>
<tr>
<th>Genotype‡</th>
<th>a/l/a</th>
<th>$A^y/a$</th>
<th>$A^y/A^y$</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hatched</td>
<td>24</td>
<td>41</td>
<td>0</td>
<td>65</td>
</tr>
<tr>
<td>Unhatched</td>
<td>4</td>
<td>9</td>
<td>14</td>
<td>27</td>
</tr>
<tr>
<td>Total</td>
<td>28 (30%)</td>
<td>50 (54%)</td>
<td>14 (15%)</td>
<td>92</td>
</tr>
</tbody>
</table>

*3.5 dpc blastocysts were obtained as described in Materials and Methods. After 3-4 days in culture, individual embryos were scored with regard to hatching from the zona pellucida and the formation of trophoblastic outgrowths.

†The agouti genotype of each embryo was inferred from the identity of a variant HindIII site at the closely linked Emv-15 locus as described in Frohman et al. (1993).

‡The recovery of $A^y/A^y$ embryos is not significantly different from a 1:2:1 distribution at the 95% confidence level ($\chi^2=4.95$, 2 degrees of freedom).
protein associated with the *lethal yellow* mutation. Two differences between the *Merc* and the *Raly* cDNA sequences would not produce significant changes in the encoded protein and may be due to the different sources used to isolate the cDNA clones; therefore, we will refer to this gene as *Raly*, subsequently. Our results confirm those of Michaud et al. (1993) and, in addition, provide a model for understanding the origin of the *Merc/agouti* fusion (Fig. 9). Finally, our expression and embryologic studies suggest that *Aγ*-associated recessive lethality is caused by loss of *Merc* gene expression.

**Relationship of *Aγ*-specific cDNAs to *agouti* genomic structure and origin of the *Aγ* mutation**

Initial studies of *agouti* cDNAs suggested the potential for a complex genomic structure in which alternative isoforms were produced with different 5′ ends, but their significance was not clear until recently. Molecular cloning and expression studies using RNA from animals that carry the wild-type light-bellied *agouti* (*Aγ*) allele have now shown that *agouti* exons 1A and 1A′ initiate isoforms only in the ventrum but throughout the entire hair growth cycle, and are located 100 kb 5′ of *agouti* exons 1B and 1C, which initiate isoforms expressed in the dorsum and the ventrum but only during the active phase (mid-anagen) of the hair cycle (see Figs 1, 9; Vrieling et al., 1994). Isoforms produced by the *agouti* (*Aγ*) allele always begin with exons 1B or 1C because the ventral-specific promoter is inactive. Of seventeen cDNA clones that we isolated from *Ay/a x* skin RNA, seven began with *agouti* exons 1B or 1C. Since there is very little RNA detectable by northern hybridization analysis from the *a x* allele, these seven cDNA clones are likely to represent normal activity of the hair cycle-specific promoter from the *Aγ* allele.

In contrast, the remaining ten cDNA clones that we isolated from *Ay/a x* skin RNA were *Merc/agouti* fusions; of these, six contained *agouti* exon 1A or *agouti* exons 1A and 1A′ due to an apparent cryptic splice acceptor in exon 1A, while four resulted from splicing of *Merc* 5′ sequences directly to *agouti* exon 2. Because none of the cDNA clones that we isolated began with *agouti* exon 1A, the ventral-specific promoter is probably not active in the *Aγ* allele. The *Aγ* mutation was one of many coat color variants bred by mouse fanciers in the 19th century; therefore its origins and relationships to current inbred strains are somewhat obscure. However, haplotype analysis of closely linked molecular markers indicates that the *Aγ* mutation is related more closely to the *A* and *Aγ* alleles than to the *nonagouti* (*a*) allele (Winkes et al., 1993). Thus, absence of

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**Fig. 7.** Morphology of 3.5 dpc embryos injected with *Merc* antisense or sense oligonucleotides at 0.5 dpc. (A) Embryos injected with the sense oligonucleotide exhibit a range of normal development from compacted morula to blastocysts. Approximately 30% of the embryos exhibit developmental abnormalities that may be a non-specific result of the injection (see Table 3). (B) Developmental abnormalities are present in nearly all the embryos injected with the antisense oligonucleotide.
ventral-specific promoter activity from the A′ allele may be explained because (1) the 120 kb A′ deletion has removed cis-regulatory sequences required for activity of the ventral-specific promoter; or (2) the allele of origin for the 120 kb A′ deletion was A; therefore the ventral-specific promoter was already inactive. Functional analysis of the ventral-specific promoter in combination with physical mapping of the A′ deletion breakpoints will help to distinguish between these possibilities.

We detected the Merc RNA in northern hybridization experiments reported here using a probe that contained exon 1Ay. In a similar experiment described in Miller et al. (1993), the 1.7 kb Merc RNA was not evident because the ‘A′-specific probe’ used in these experiments comprised agouti exons 1A and 1A′. As described above, these exons are found normally only in ventral-specific agouti isoforms; therefore, exons 1A and 1A′ appear specific for the A′ cDNA when analyzed opposite agouti alleles where the ventral-specific promoter is inactive such as A or a. Exons 1A and 1A′ were also present in several A′-specific cDNAs isolated by Michaud et al. (1993).

Pleiotropy associated with heterozygosity for A′

The Melcagouti fusion RNAs and the normal Merc RNA are both expressed in every adult tissue that we have examined, suggesting that the 120 kb A′ deletion has not altered regulatory elements which normally direct Merc expression. The fusion RNAs are capable of encoding a normal agouti protein, suggesting that ectopic expression of this protein is responsible for the pleiotropic effects associated with heterozygosity for A′ (Carpenter and Mayer, 1958; Castle, 1941; Danforth, 1927; Granholm and Dickens, 1986; Granholm et al., 1986; Heston and Deringer, 1947; Heston and Vlahakis, 1961; Vlahakis and Heston, 1963). Alternative explanations for the pleiotropic effects, including the production of a novel fusion protein or the action of closely linked genes, are unlikely since the pleiotropic phenotype is observed in mice that carry the viable yellow (A′y) mutation (Dickie, 1962; Wolff et al., 1986), in which an agouti fusion RNA is also expressed ubiquitously (Yen et al., 1994), and in which mutant animals can be compared to coisogenic non-mutant littermates.

There are several possibilities to explain how ectopic expression of the normal agouti protein could lead to obesity and increased tumor susceptibility, including antagonism of melanocortin receptors sequestration of melanocortins, or activation of an as yet unidentified agouti receptor (Conklin and Bourne, 1993; Jackson, 1993) [reviewed in Yen et al. (1994)]. Experiments with adipose tissue transplants (Meade et al., 1979) and aggregation chimeras (Barsh et al., 1990) suggest

**Table 3. Development in vitro of 1-cell embryos injected with Merc antisense or control oligonucleotides, with or without a Merc expression vector**

<table>
<thead>
<tr>
<th>Injected DNA</th>
<th>Dilution of Oligonucleotide†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>(1) Control “scrambled”</td>
<td>5/12 (42%)</td>
</tr>
<tr>
<td>(2) Sense</td>
<td>4/22 (18%)</td>
</tr>
<tr>
<td>(3) Antisense</td>
<td>1/36 (2.8%)</td>
</tr>
<tr>
<td>Chi-square vs. (1+2)‡</td>
<td>6.20; p=0.013</td>
</tr>
<tr>
<td>(4) Antisense + Merc transgene</td>
<td>2/21(9.5%)</td>
</tr>
<tr>
<td>Chi-square vs. (1+2)‡</td>
<td>1.4; p=0.24</td>
</tr>
<tr>
<td>Chi-square vs. (3)</td>
<td>0.24; p=0.63</td>
</tr>
<tr>
<td>(5) Antisense + control transgene</td>
<td>7/21 (33%)</td>
</tr>
</tbody>
</table>

*Data are given as No. of embryos developing into blastocysts by 4.5 dpc/No. of embryos injected (percent). The data shown include the results depicted in Figure 8 and represent the results of four separate experiments.

†A solution of 10 mM Tris, pH7.5; 0.1mM EDTA that contained either oligonucleotide or oligonucleotide + linear DNA was injected into each embryo. Therefore, dilutions of 1, 1:50, and 1:2500 correspond approximately to 5×10^5, 1×10^5, or 2×10^5 molecules of sense or antisense oligonucleotide placed into microdrop culture as described in Materials and Methods. Embryos were scored daily and categorized within the five conditions shown on the right. Bars represent percentages (shown on the abscissa) of embryos in each condition on each day. The results shown represent the sum of two different experiments, in which a total of 150 embryos were injected with 5×10^5, 1×10^5, or 2×10^5 molecules of antisense or sense oligonucleotide.

![Fig. 8. Development of embryos injected with Merc antisense or sense oligonucleotides between 0.5 dpc and 4.5 dpc.](image-url)
that A\textsuperscript{-} induced obesity is cell non-autonomous; however, parabiosis experiments demonstrate that the factors that mediate A\textsuperscript{-} induced obesity are not transmitted through the circulation (Wolff, 1963). Thus, like the action of agouti in hair follicles (Silvers, 1958, 1961), agouti-induced obesity may be mediated by a diffusible factor with a limited radius of action.

**A\textsuperscript{y}-associated embryonic lethality**

In contrast to the pleiotropic effects associated with heterozygosity for A\textsuperscript{y}, several observations have suggested that lethality of A\textsuperscript{Y}/A\textsuperscript{y} embryos is not due to altered expression of the *agouti* gene (Barsh and Epstein, 1989; Barsh et al., 1990; Lyon et al., 1985). Our results provide a molecular explanation for these observations, since the 120 kb A\textsuperscript{y} deletion produces two distinct genetic lesions; a neomorphic effect caused by ubiquitous expression of the *Mercagouti* fusion RNA, and an amorphic effect due to the loss of *Merc*. Although the expression of other genes besides *Merc* and *agouti* may be affected by the A\textsuperscript{y} deletion, injection of 1-cell embryos with *Merc* antisense oligonucleotides prevents development to the blastocyst stage, suggesting that loss of *Merc* gene expression is sufficient to explain A\textsuperscript{y}-associated lethality. Of three other recessive lethal *agouti* mutations that have been studied, only one, *nonagouti lethal* (a\textsuperscript{d}), fails to complement A\textsuperscript{y} (Lyon et al., 1985). Genetic and molecular studies of a\textsuperscript{d} indicate it is a deletion that removes several genes (Barsh and Epstein, 1989; Lyon et al., 1985), and mapping of the a\textsuperscript{d} proximal breakpoint may help to refine further the physical boundaries of the A\textsuperscript{y} recessive lethal complementation group, and to delimit regions required for normal *Merc* gene expression.

Functional and/or immunologic assays for *Merc* will be required to determine the degree to which its expression is affected by injection of antisense oligonucleotides but, in general, inhibition of gene expression using antisense approaches is usually not absolute, and preimplantation development may therefore be sensitive to dosage of the *Merc* protein. Heterozygosity for A\textsuperscript{y} has no obvious effect on viability; however, increased expression of *agouti* in these animals may mask subtle effects on growth caused by a 50% reduction in *Merc* gene expression since a\textsuperscript{d} animals are slightly reduced in size compared to a\textsuperscript{a} animals (unpublished observations). Subtle effects of reduced *Merc* gene dosage on growth and development may also account for the observation that A\textsuperscript{Y}/a cells are recovered less frequently than expected in aggregation chimeras between A/A embryos and those derived from an A\textsuperscript{Y}/a × A\textsuperscript{Y}/a intercross (Barsh et al., 1990).

Previous studies of embryos derived from A\textsuperscript{y}− × A\textsuperscript{y}− intercrosses have suggested that homozygosity for A\textsuperscript{y} may lead to a wide variety of developmental defects (Calarco and Pedersen, 1976; Eaton and Green, 1963; Johnson and Granholm, 1978; Papaioannou, 1988; Papaioannou and Gardner, 1979, 1992; Pedersen, 1974). Although these previous studies were complicated by an inability to determine the genotype of morphologically abnormal embryos, our results confirm that A\textsuperscript{Y}/A\textsuperscript{y} embryos can develop to the blastocyst stage yet fail to hatch when placed into culture or into diapause.

**Function of the Merc gene**

Embryos injected with *Merc* antisense oligonucleotides rarely develop to the blastocyst stage, and thus are more severely affected than A\textsuperscript{Y}/A\textsuperscript{y} embryos. Injection of control sense or ‘scrambled’ oligonucleotides did not prevent blastocyst development, which suggests that the effects that we observed after injection with *Merc* antisense oligonucleotides are likely to be due to loss of *Merc* gene expression. Although expression of RNA in eggs and in embryos is difficult to quantitate, our results clearly indicate that *Merc* RNA is present in unfertilized eggs, possibly at much higher amounts than in blastocysts. Thus, maternal *Merc* RNA and/or protein in the A\textsuperscript{Y}/A\textsuperscript{y} embryos may provide a ‘sparing’ effect compared to non-mutant embryos injected with *Merc* antisense oligonucleotides. These results are also consistent with an inability to rescue A\textsuperscript{Y}/A\textsuperscript{y} cells in aggregation chimeras (Barsh et al., 1990), and suggest that loss of *Merc* gene expression in preimplantation embryos is a cell-lethal. It remains to be determined, however, whether *Merc* expression is required for cell viability later in development, especially given the evidence that A\textsuperscript{Y}/A\textsuperscript{y} inner cell mass can be rescued in blastocyst injection chimeras (Papaioannou and Gardner, 1979). It is possible that the *Merc* gene product plays a specialized role in metabolism of maternal stored RNAs, and that, later in development, general roles for hnRNPs. Although oogenesis proceeds differently in mammals and invertebrates, it is intriguing that a recently described mutation that interferes with dorsal/ventral axis formation during *Drosophila* oogenesis, *squid* (Kelley, 1993), encodes one of the major *Drosophila* hnRNPs (Kelley, 1993; Matunis et al., 1992).
The predicted Merc protein is 80% identical to human hnRNP C over the first 90 amino acids. This region contains a well-characterized RNA-binding domain found in many hnRNPs which assembles into a four-stranded beta-sheet and binds to homopolymeric uridine tracts in vitro (Burd et al., 1989; Gorlach et al., 1992; Wittekind et al., 1992). Although the carboxyl terminus of the predicted Merc protein is very different from hnRNP C, it contains several regions rich in glycine and serine, which are found in other hnRNPs (Burd et al., 1989), and the Merc protein product is likely to function as an hnRNP in cells. In vitro and cell culture experiments suggest that hnRNPs mediate processing and/or transport of newly synthesized nuclear RNA in most eukaryotic cells. Embryonic lethality due to absence of Merc expression in mice is somewhat surprising given the large number of hnRNPs in most organisms, and the potential for functional redundancy. Experiments directed at identification and isolation of Merc homologs in other organisms may help shed light on the possibility that Merc encodes an hnRNP used specifically for the processing and/or transport of maternally expressed RNA.

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