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Reduced pigmentation (*rp*), a new coat colour gene with effects on kidney lysosomal glycosidases in the mouse

By S. GIBB, E. M. HÅKANSSON*, L.-G. LUNDIN* and J. G. M. SHIRE†

*Institute of Genetics, University of Glasgow, Glasgow G11 5JS, Scotland, * Institute for Medical Genetics, V. Agatan 24, S 75220 Uppsala, Sweden and † Department of Biology, University of Essex, Colchester CO4 3SQ, England*

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SUMMARY

A spontaneous autosomal mutation in C57BL/Tb mice, provisionally called reduced pigmentation, symbol *rp*, has pronounced effects on three kidney lysosomal glycosidase activities. Homozygous *rprp* mice have significantly higher activities of β-galactosidase, β-glucuronidase and *N*-acetyl-β-hexosaminidase than their heterozygous litter-mates. Homozygotes have light ears and tails, diluted fur and dark eyes. The mutation is not allelic to any known to affect lysosomal functions, or to a number of pigmentation variants with similar phenotypic effects. The locus is on chromosome 7.

1. INTRODUCTION

Alleles at a number of loci reduce the intensity of skin and hair pigmentation in mice (Wolfe & Coleman, 1966; Searle, 1968; Silvers, 1979). Several of these loci can be assigned to one or other of a number of groups of mimic genes which produce almost identical phenotypes (Green, 1966). These mimic genes are usually on different chromosomes and may represent derivatives of a common ancestral gene (Lundin, 1979). These groups of mimics include dilute (*d*) and leaden (*ln*), ruby-eye (*ru*) and ruby-eye-2 (*ru-2*) (Eicher, 1970), pale ears (*ep*) and light ears (*le*) (Lane & Green, 1967), pallid (*pa*), pearl (*pe*) and muted (*mu*) (Searle, 1968), and beige (*bg*) and buff (*bf*) (Håkansson & Lundin, 1977).

An effect of the coat colour locus, beige, on kidney lysosomal glycosidase activities was reported and it was shown that beige coloured animals have higher activities than their black litter-mates (Brandt, Elliott & Swank, 1975; Brandt & Swank, 1976). A similar effect of the light-ear locus was described by Meisler (1978). Håkansson & Lundin (1977) found likewise that the buff locus had a strong effect on three lysosomal glycosidase activities. Later Swank and others (Novak & Swank, 1979; Swank, Novak, Brandt & Skudlarek, 1978) screened 20 coat colour mutants for possible effects on lysosomal enzymes. This resulted in the identification of five additional loci, pallid, pearl, pale-ear, ruby and ruby-2 (*ru-2*^{mr}), with such an effect.

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This paper describes the occurrence, inheritance, and phenotype of a new mutation which has strong similarities with many of the diluting genes described above including an effect on lysosomal enzymes. We suggest the mutation be called 'reduced pigmentation' (symbol *rp*), and that it belongs to the select group of mutations that affect lysosomal function as well as coat colour.

2. MATERIALS AND METHODS

(i) *Animals*

Mice were bred and maintained under controlled conditions. Those in Glasgow were fed Oxoid Breeding Diet whilst those in Uppsala were given Anticimex Breeders Chow. The C57BL/Tb mice were obtained from the Department of Zoology, University of Glasgow, several years before the unusual phenotype appeared. The C57BL/10ScSn mice came from the Medical Research Council Laboratory Animal Centre at Carshalton in England. The DBA/2Kl strain came from the laboratory of Professor G. Klein in Stockholm, and the DBA/2J mice from the Jackson Laboratory.

DBA/2J mice supplied the dilute, *d*, and brown, *b*, alleles used in the complementation tests. The albino, *c*, and pinkeye, *p*, alleles came from 129/RrJ mice. C57LN from Carshalton were used as the source of the leaden (*ln*) mutation. Misty (*m*), muted (*mu*), ruby (*ru*), ruby-2 (*ru-2*), pale ear (*ep*) and pearl (*pe*) mutants were kindly supplied by Dr M. F. Lyon, MRC Radiobiology Unit, Harwell, England. The buff (*bf*), beige (*bg*) and pallid (*pa*) mutants were obtained from the Jackson Laboratory, Bar Harbor, Maine.

(ii) *Mapping*

Linkage testing was carried out by crossing C57BL mice homozygous for the new mutation to DBA/2Kl mice and then backcrossing the resultant heterozygotes to C57BL mice homozygous for the mutation. These backcross mice were scored for coat colour and for a number of marker genes. Standard detection methods were used to score the segregation at the haemoglobin b (*Hbb*: Petras & Martin, 1969), esterase 3 (*Es-3*: Ruddle & Roderick, 1968), glucosephosphateisomerase (*Gpi*: Carter & Parr, 1967; Shows & Ruddle, 1968), isocitratedehydrogenase (*Idh-1*: Ashton & Braden, 1961; Henderson, 1965) and malatedehydrogenase (*Mod-1*: Shows & Ruddle, 1968) loci.

(iii) *Lysosomal enzyme assays*

The animals used were between 65 and 76 days old and were killed, by cervical dislocation, during a limited period of time from late November to early March, always between 9 and 12 noon. Blood samples were taken from the orbital sinus and the kidneys and liver were rapidly excised. They were weighed and kept frozen until homogenized. Homogenization was done with a Potter–Elvehjem glass–teflon homogenizer in distilled water, twice the sample weight. The homogenizer was chilled in an ice bath during this procedure. After freezing and thawing three

times, the homogenates were centrifuged at 500 *g* for 30 min in a refrigerated centrifuge. Supernatants were stored at −25 °C until assayed. The three glycosidase activities β-galactosidase, β-glucuronidase and *N*-acetyl-β-hexosaminidase were assayed with p-nitrophenyl-β-galactoside, phenolpthalein glucuronic acid and p-nitrophenyl-β-glucosaminide as substrates (Håkansson & Lundin, 1977).

The lysosomes of mast cells in the intestinal mesentery were stained with toluidine blue after fixation in 10% formalin (Kitamura, Matsuda & Hatanaka, 1979).

(iv) *Hormone treatment*

5α-Dihydrotestosterone (DHT) has been shown to be the most effective inducer of β-glucuronidase activity (Swank, Paigen & Ganschow, 1973). In these experiments, 5α-dihydrotestosterone (5α-androstan-17β-ol-3-one) from Sigma Chemicals was used. A 50 mg/ml concentration was suspended in olive oil with an ice-chilled homogenizer. Females were injected subcutaneously with 0·2, 0·2, and 0·1 ml, respectively, on days 0, 1 and 4 and were killed on day 7.

(v) *Hair phenotype*

Hairs were gently plucked from the backs of young adult animals. They were dehydrated in alcohol, cleared in xylene and mounted in DPX. The morphology of the hair and its pigment is described with the terms defined by Russell (1949).

3. RESULTS

(i) *Origin of the mutant mice*

A pink-eyed male with cream coloured fur appeared in the Glasgow colony of C57BL/Tb mice in 1975. This mouse was fully fertile and was shown to be homozygous for an allele at the pink-eye (*p*), locus. The mouse was mated to a DBA female and the black, non-agouti, F_1 offspring intercrossed to produce an F2 segregating for several coat-colour genes for use in undergraduate classes. Some of these F_2 matings produced litters in which there was an unexpected phenotype: mice with very pale grey fur and dark eyes. At about this time a brother–sister mating of C57BL/Tb mice closely related to the cream mouse produced litters containing mice with grey fur and dark eyes as well as mice with the normal black non-agouti colouring. This suggests that the mutant allele was present in several members of the C57BL/Tb strain at that time and that the mutational event giving rise to *rp* did not occur in the cream-coloured male. The mutant was transferred to a predominantly C57BL/10ScSn background by two cycles of backcrossing.

(ii) *External phenotype of the mutant mice*

Mutant C57BL mice have grey fur, very much the same colour as that of black, non-agouti, dilute homozygotes. The mutant mice have dark eyes. The ears of the mutant mice are pale and not grey like those of *BB aa dd* and *BB aa ln ln* homozygotes. The tail is the same colour as the ears in the mutant. The colour of the fur, skin and eyes do not appear to alter with age.

The amount of pigment in all four kinds of body hair is much less than that

found in non-mutant C57BL mice, and seems to be less than that found in black, non-agouti, dilute homozygotes. Cortical and medullary pigment are both reduced. The pigment granules are smaller in the mutant mice (Plate 1). The granules are not clumped together, unlike many of those in the hairs of mice homozygous for the dilute mutation (Russell, 1949).

The fertility and viability of mutant mice of both sexes seem to be the same as that of non-mutant C57BL/10ScSn mice. We have no evidence that the life-span of the mutants is any different to that of non-mutant C57BL mice.

(iii) *Genetic investigations*

The offspring of all matings between mutant mice and unrelated C57BL or DBA mice were black and non-agouti, whether crosses were made with mutant males or with mutant females. Table 1 summarizes the results of matings of presumed heterozygotes to mice with the mutant phenotype. In all cases approximately equal numbers of animals with mutant and non-mutant phenotypes were found. There was no significant difference in the ratio of the two phenotypes between male and female backcross mice.

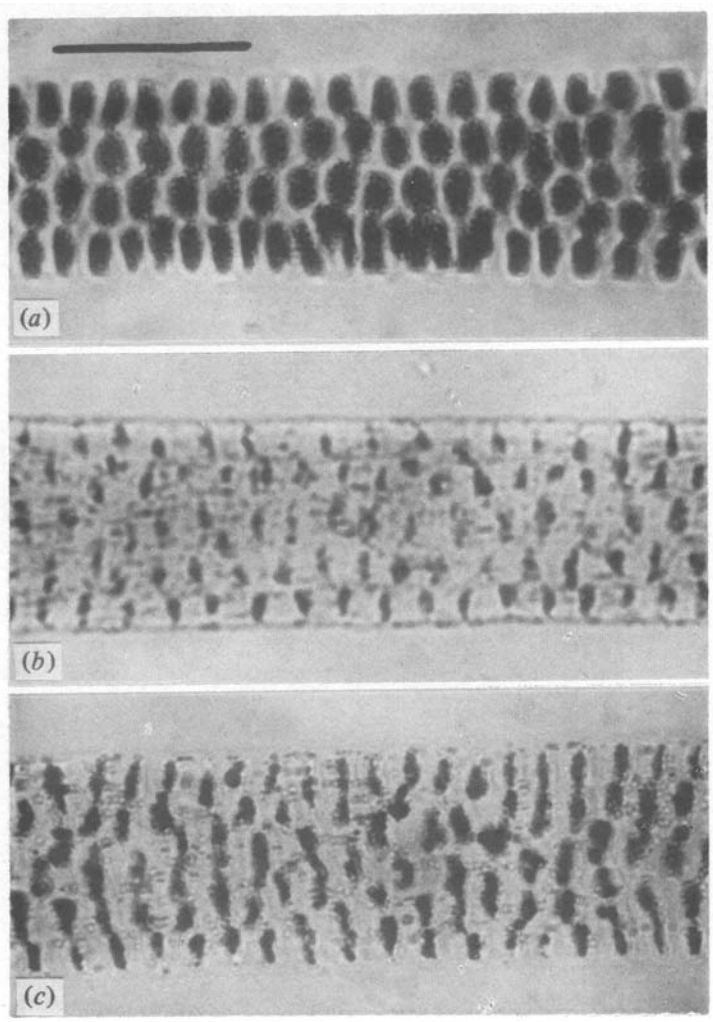
Table 1. *Segregation data for crosses involving heterozygotes*

Cross	Source of heterozygote	Total progeny	Black	Grey	Ratio	χ^2
rp/+ × *rprp*	C57	77	42	35	1:1	0·64
rp/+ × *rprp*	(C57 × DBA)F1	204	107	97	1:1	0·49
rp/+ × *rp*/+	(C57 × DBA)F1	30	21	9	3:1	0·40

Complementation tests were carried out with a number of genetic variants affecting coat colour. They were negative for brown (*b*), buff (*bf*), beige (*bg*), albino (*c*), dilute (*d*), pale ears (*ep*), leaden (*ln*), misty (*m*), muted (*mu*), pink-eyed dilution (*p*), pallid (*pa*), pearl (*pe*), ruby-eye (*ru*) and ruby-eye-2 (*ru-2*).

In F_2 crosses of C57BL with DBA the mutation and dilute interacted, when homozygous, to give mice with almost white coats and dark eyes. Such mice that had one or more black alleles at the *B* locus were greyer than those that had brown alleles. Observations on crosses suggest that it is possible that there may be some interaction between beige and reduced pigmentation with effects on other systems besides the skin.

Linkage studies on backcross mice, summarized in Table 2, showed that the *rp* locus was unlinked to loci of chromosomes 1, 9 and 11. The mutant locus showed very significant linkage to *Gpi* at the proximal end of chromosome 7 but insignificant linkage to the more distally placed *Hbb* locus. The map distances calculated for males and females separately were very similar. The distances calculated from all the backcross data were additive: *rp-Gpi* 10·8 ± 2·2 %, *Gpi-Hbb* 36·3 ± 3·4 % and *rp-Hbb* 46·6 ± 3·5 %, placing *rp* between *Gpi* and the centromere. One putative double crossover was recorded, a male mouse with grey fur, heterozygous at the *Gpi* locus, but homozygous at the *Hbb* locus.



Microphotographs of guard hairs from three genotypes of mice. The bar indicates 10 μm. (*a*) C57 with the normal, non-agouti, genotype, *aa* rp^+ rp^+. (*b*) C57 homozygous for reduced pigmentation, *aa rp rp*. (*c*) Non-agouti dilute, *aa dd* rp^+ rp^+ *Bb*, from the backcross of C57 to DBA.

Table 2. *Linkage tests of reduced pigmentation*, rp, *with five marker loci*

	Chromosome	Parental phenotypes		Recombinant phenotypes		
		rp^+	*rprp*	rp^+	*rprp*	$\chi^2(1)$
Isocitratedehydrogenase *Idh-1*	1	31	15	20	26	0·01
Malicdehydrogenase *Mod-1*	9	47	30	44	38	0·14
Esterase-3 *Es-3*	11	21	12	24	18	0·80
Haemoglobin b *Hbb*	7	54	56	53	41	1·06
Glucosephosphateisomerase *Gpi*	7	94	88	13	9	122·5

Linkage tested by contingency χ^2, corrected for continuity.

(iv) *Lysosomal studies*

The mean values of the activities of three lysosomal enzymes in kidneys from male and female mice are shown in Table 3. The activities of the enzymes were higher in males than in the corresponding females. Treatment of female mice with DHT significantly increased ($P < 0{\cdot}01$) the activities of all three enzymes above those found in untreated females of the same genotype, with the single exception of galactosidase in *rprp* C57BL females. The rise in β-glucuronidase following DHT treatment was about tenfold. In 13 out of 15 comparisons between black animals and their grey litter-mates the mean activities of the grey groups were significantly higher than in the corresponding black groups. The only exceptions were the hexosaminidase activities in males and females of the C57BL strain.

The mesenteric mast cells of *rprp* mice contained lysosomal inclusions which were similar in size and number to those of rp^+rp^+ mice.

4. DISCUSSION

The segregation data indicate that the mutation is autosomal and recessive. Reduced pigmentation (*rp*) is suggested as the name for this mutation. It does not complement with *b*, *bf*, *bg*, *c*, *d*, *ep*, *ln*, *m*, *mu*, *p*, *pa*, *pe*, *ru* or *ru-2*. M. Meisler (pers. comm.) has found that mice heterozygous for *rp* and *le* were black. The mutation, when homozygous, reduces the pigmentation of non-agouti mice, unlike grizzled (*gr*, Green, 1966). Male and female homozygotes are fully viable and fertile, unlike homozygotes for grey–lethal (*gl*), gunmetal (*gm*) and taupe (*tp*), (Green, 1966). Mocha (*mh*, Lane & Deol, 1974) and muted (*mu*, Lyon & Meredith, 1969) homozygotes show defects in balancing behaviour and otolith structure not found in *rprp* mice. The degree of pigment reduction does not change with age in *rp*, while such changes are a marked feature of greying-with-age (*Ga*, Kirby, 1974) and Ochre (*Och*). Homozygotes for underwhite (*uw*) have white underfur or belly spots (Green, 1966), which are not present on *rprp* mice, and do not have altered levels of lysosomal enzymes (Swank *et al.* 1978). Four mutants that bear some resemblance to *rp* and that have not been tested for complementation with *rp* have been described in the literature. Ashen (*ash*) resembles dilute in its effects on pigment granules and in the absence of any effects on ear pigmentation or

Table 3. *The activity of three lysosomal enzymes in the kidneys of* rprp *and* rp/+ *mice*

Sex	Coat Colour	Number	Galactosidase mean ± SD	Glucuronidase mean ± SD	Hexosaminidase mean ± SD
			(C57 × DBA) × C57		
M	Black	46	7·44 ± 1·33	2·81 ± 1·24	27·25 ± 4·17
M	Grey	33	12·67 ± 2·54***	3·55 ± 1·53*	30·26 ± 3·53**
F	Black	32	6·56 ± 1·36	1·47 ± 0·13	18·48 ± 2·40
F	Grey	22	8·88 ± 2·29***	1·89 ± 0·27***	20·89 ± 2·84**
			(C57 × DBA) × C57 DHT treated		
F	Black	13	8·27 ± 1·46	14·53 ± 7·01	24·51 ± 2·68
F	Grey	13	10·00 ± 1·65**	23·39 ± 9·64*	26·76 ± 2·75*
			C57 *rp/+* × C57 *rprp*		
M	Black	13	9·91 ± 1·11	1·94 ± 0·45	31·09 ± 3·94
M	Grey	12	16·07 ± 2·40***	2·54 ± 0·20**	31·57 ± 4·23
F	Black	15	8·73 ± 1·18	1·52 ± 0·12	22·49 ± 4·88
F	Grey	19	12·18 ± 1·33***	1·98 ± 0·25***	20·71 ± 3·53

Activities expressed as mg product liberated per h per g wet weight of tissue. Probability for t test of difference between black and grey groups: *** $P < 0{\cdot}001$, ** $P < 0{\cdot}01$, * $P < 0{\cdot}05$.

lysosomal enzyme activities (Lane & Womack, 1979). Sepia (*sea*) resembles beige in that *BB aa sea sea* mice are brownish (Sweet & Lane, 1977) whereas *BB aa rprp* mice are grey. The descriptions of Slaty (*slt*, Green 1972) and dilution-Peru (*dp*, Wallace, 1971) do not allow them to be unequivocally distinguished from the mutation described in this paper. The chromosomal locations of these four loci, on chromosomes 1, 9, 14 and 15 respectively, do distinguish them clearly from *rp* which has been mapped to chromosome 7.

Taupe, (*tp*), is a mutation that dilutes the pigment of both fur and ears and is linked to pink-eyed dilution-*p*, on chromosome 7 (Fielder, 1952). However, taupe has been placed only 8 mapunits from *Hbb* (Davisson & Roderick, 1979), unlike *rp*. Taupe females also have defective nipples and are unable to rear their young (Fielder, 1952), whilst *rprp* females rear their young without difficulty. Another dilution locus (*ru-2*), which is known to affect the activity of lysosomal enzymes (Swank *et al.* 1978), is about 11 map units distal to *Gpi* and is not allelic with *rp*. Thus, including pink-eyed dilution, there may well be four loci with alleles that dilute coat colour on chromosome 7.

There is a pronounced effect of the *rp* allele on the three lysosomal glycosidase activities in kidney. The magnitude of the differences between *rprp* and black animals is similar to that seen in crosses with the buff locus (Håkansson & Lundin, 1977) and somewhat smaller than that seen in crosses with the beige locus (Brandt *et al.* 1975; Brandt & Swank, 1976). As with beige the phenotypic difference was enhanced in testosterone treated females. A similar effect of the light-ear gene reported by Meisler (1978) can be compared, at least partly, with the other three genes in this respect. Homozygous *le le* males and testosterone treated females had kidney galactosidase activities 4-fold higher than the controls whereas *bf bf* and *rp rp* males have 2-fold and 60 % higher activities than black animals, respectively.

Male kidney activities are higher than the corresponding female activities for all three enzymes. DHT treatment of female mice increased the activities in the backcrosses to DBA (Table 3). However, we have repeatedly found that C57BL and C57 × DBA F_1 mice respond with decreased or only slightly increased kidney galactosidase and hexosaminidase activities after DHT injections whereas DBA and backcross animals usually respond with moderately increased activities after this treatment. In view of the findings concerning the genetic control of secretion of lysosomal enzymes (Meisler, 1978; Watson & Paigen, 1978) it seems likely that there is a different effect of androgens on this secretion in DBA and C57BL mice. Indeed DBA and C57 differ in a number of aspects of androgen metabolism (Wilson, Erdos, Dunn & Wilson, 1977; Bartke & Shire, 1972; Shire, 1979).

The differences between *rprp* and black mice in the backcrosses to DBA are in reality even more significant than they would appear from Table 3. This is due to segregation of the genes for activity levels of β-galactosidase, *Bgs* (Lundin & Seyedyazdani, 1973) and β-glucuronidase, *Gur*, (Swank *et al.* 1973). Differences between the coat colour groups tend to be obscured by these two variables and perhaps also by endogenous physiological events such as the sexual cycle.

Genes that affect pigmentation fall into two classes: a large one in which

lysosomal enzymes are unaffected and a smaller one in which they are elevated, at least in kidney cells. The small group at present contains, *bf*, *bg*, *ep*, *le*, *pa*, *pe*, *ru*, *ru-2* and *rp*. Some members of the larger class fall into groups of mimic genes, such as dilute (*d*), leaden (*ln*) and ashen (*ash*). Buff and beige form such a pair of mimics within the loci known to affect lysosomal function as do ruby and ruby-2 and also light ears and pale ears. The pigmentation of *rp rp* mice closely resembles those of mice homozygous for either *le* or *ep*, suggesting that *rp* belongs in this mimic group. Pallid (*pa*), in which the otoliths are affected, and pearl (*pe*) are not mimics of each other or of any of the other genes so far known to have effects on lysosomal enzymes.

For the main mutants affecting lysosomal function the major effects are limited to the kidney and only smaller changes are found in other tissues even though a structural analysis of beige mice showed the lysosomes to be greatly enlarged in liver, leucocytes (Windhorst & Padgett, 1973) and mast cells (Kitamura *et al.* 1979). The absence of any differences in the mast cells of *rprp* mice suggests that the effects of the *rp* locus may be limited to lysosomes in the kidney and to melanosomes.

The availability of variants at nine loci that affect lysosomal functions, together with variation in some of the lysosomal enzymes (Swank *et al.* 1978; Håkansson & Lundin, 1977), should enable the analysis of the differentiation and regulation of lysosomes to proceed rapidly.

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