

Gene expression at the pink-eyed dilution (*p*) locus in the mouse is confirmed to be pigment cell autonomous using recombinant embryonic skin grafts

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SUMMARY

Sash (*W^{sh}*), a viable and fully fertile allele of the dominant spotting (*W*) locus (Lyon & Glenister, 1982) has been used in a modified test system to investigate the site of gene expression at the pink-eyed dilution (*p*) locus. Reciprocal recombinant epidermal//dermal skin grafts were constructed from 13-day embryonic skin of *p* and *W^{sh}* homozygotes. Thus in the reciprocal experiments pink-eyed dilution melanocytes were exposed to any environmental influence from the wild-type allele of the *p* locus in either the epidermis (when *W^{sh}W^{sh}*) or the dermis (when *W^{sh}W^{sh}*). The hair pigmentation of the grafts recovered after three weeks beneath the testicular tunica of adult male mice was always typical of the *p* phenotype showing that *p* is melanocyte autonomous. This result was supported by experiments using a modification of Mayer's (1965) neural crest grafting technique and the construction of 14-day recombinant skin grafts.

Sash (*W^{sh}W^{sh}*) epidermis can support melanocyte differentiation and pigment production but lacks functional melanocytes. The advantages of *W^{sh}* in experimental systems for testing the site of pigment gene expression have been demonstrated.

Control experiments confirmed the dermal influence of agouti (*A*) over non-agouti (*a*) epidermis but non-agouti dermis did not overrule agouti pink-eyed dilution (*AA pp*) epidermis suggesting an epistatic effect of *p* in the melanocyte.

INTRODUCTION

In the mouse, genes involved in the production of pigment have been shown to be expressed within the melanocyte itself, or through an interaction between the melanocyte and the cells of the local tissue environment (see Silvers, 1979; Poole & Silvers, 1980). They therefore provide a useful model for investigating differential gene expression during development. In addition, loci known to be expressed within the melanocyte (i.e. cell autonomous) could be useful in the development of *in vivo* somatic cell mutational testing systems (Searle, 1977; 1978*a*; Searle & Stephenson, 1982; Stephenson, 1983).

The pink-eyed dilution (*p*) mutant, derived from the mouse Fancy (Grüneberg, 1952), has pink eyes and reduced pigment intensity of the coat (Searle, 1968;

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Key words: agouti, differential gene expression, dominant spotting locus, epidermal/dermal recombinant grafts, pink-eyed dilution (*p*), sash (*W^{sh}*).

Silvers, 1979; Green, 1981). In the hairs, granules of black (eumelanin) pigmentation are greatly reduced in size and distribution (Russell, 1949) producing a light-grey phenotype on a non-agouti (*a*) background. Phaeomelanin pigmentation is only slightly affected, so on an agouti (*A*) background *pp* has a pale yellow phenotype. Indirect evidence, including the failure of invading host melanocytes to respond to the influence of a skin graft homozygous for *p* (Silvers, 1958*a*), suggests that gene expression at the *p* locus is cell autonomous. If autonomous, it could be used in a somatic cell mutational testing system to detect reverse mutational events (Searle, 1977).

Several methods have been developed to investigate the site of pigment gene expression (see Poole & Silvers, 1980). These methods involve the examination of hair pigmentation produced when melanocytes, carrying the mutant allele at the locus under investigation, are exposed to the influence of skin (or its components) whose cells carry the normal wild-type allele. In the technique described by Mayer (1965) the melanocytes originate from 8 to 9-day-old neural crest material. The tissue environment is provided by 12-day-old embryonic skin, implanted at the same time as the neural crest material. The technique used by Poole & Silvers (1976) relies on the observation that melanocytes will migrate from the dermis into the epidermis of 12- or 13-day embryonic skin grafts if the epidermal melanocyte population is absent or deficient (Mayer, 1973*a,b*). Poole & Silvers (1976) took 12-day embryonic dermis and combined it with epidermis of the same age to test whether the epidermal genotype had an effect on melanocyte gene expression. Possible dermal influence was determined by constructing 14-day recombinant skin grafts in which the melanocytes are already resident in the epidermis prior to follicle formation. In both studies, pigmentation by indigenous melanocytes was eliminated by the use of albino mutants. Other investigations (Silvers, 1958*a,b*) have exploited spotting mutants (e.g. White, *Mi^{wh}*) to remove the indigenous melanocyte population completely.

Skin from mutant alleles at the dominant spotting (*W*) locus could also be used as:— (i) there is an absence of follicular melanocytes (Silvers, 1953; 1958*b*); and, (ii) gene expression is probably cell autonomous (Mayer & Green, 1968; Mayer, 1979). Although homozygotes and heterozygotes for dominant alleles (e.g. *W^vW^v*, *WW^v*) have a black-eyed white phenotype (i.e. lacking neural-crest-derived melanocytes) they are either sterile or die at a young age (Silvers, 1979). Recently, Lyon & Glenister (1982) described a dominant allele (sash, *W^{sh}*) with a homozygous black-eyed white phenotype which is both viable and fully fertile. The use of sash homozygotes would eliminate problems of identification encountered by Mayer (1973*b*) who crossed heterozygotes (*W+* × *W^v+*) to obtain embryonic skin genetically devoid of melanocytes. In the present study, 13-day reciprocal recombinant skin grafts between sash and pink-eyed dilution homozygotes were constructed to investigate the site of pigment gene expression at the *p* locus. 14-day recombinant skin grafts, using albino homozygotes to provide the genetically unpigmented skin, were used to test for possible dermal influence on gene expression at the *p* locus.

A modification of the neural-crest-grafting technique described by Mayer (1965) using mouse testis as the culture site, is also presented.

METHODS

The following stocks and strains, available at the MRC Radiobiology Unit, Harwell, were used in the present study: BALB/c (*bb*, *cc*); JU/FaCt (*aa*, *cc*); JU/FaCt-C (*aa*); CBA/CaH - +*p* (*pp* × *p*+) and PM (*W^{sh}W^{sh}* or *W^{sh}go bf/W^{sh}go bf*). All the stocks are inbred except for PM which is maintained by random breeding within a closed colony on a 3H1 (F₁ C3H/HeH × 101/H) background.

Embryos of known age were obtained from timed matings, counting the morning on which the vaginal plug was observed as day zero. Pregnant females on either the 9th and 12th, 13th or 14th day of gestation were killed by cervical dislocation. The intact uterus was dissected out and placed in Tyrode's salt solution (Difco Laboratories). Embryos were removed and placed in fresh Tyrode's salt solution at 4°C.

13- and 14-day recombinant skin grafts were made by the method described by Mayer & Fishbane (1972). Dorsal skin from the flanks between the developing limbs was removed, and placed in a 1% Trypsin (Sigma) solution dissolved in Tyrode's salt solution without Ca²⁺ and Mg²⁺ (Difco Laboratories) at 4°C to separate dermis from epidermis. Once separation was complete, further digestion of the tissue was stopped by the addition of 40% horse serum (Gibco-Bio-Cult) in Tyrode's salt solution. The time taken for the epidermis to separate from the dermis was dependent upon the age of the skin. The epidermis was transferred and stretched out on to semisolid culture medium containing:— Eagles medium (Gibco-Bio-Cult), penicillin/streptomycin (Gibco-Bio-Cult), horse serum and 0.5% agar. The dermis was then placed on top of the epidermis, excess fluid was removed and the tissue cultured for 12–24 h at 37°C in a CO₂-enriched environment.

The neural-crest-grafting technique was modified from the method described by Mayer (1965) by culturing the tissues together *in vitro* overnight and then transplanting to the mouse testes. Neural tube material was removed from the open end of the neural fold of 9-day-old mouse embryos with finely drawn out glass needles. After treatment with 1% trypsin in Tyrode's salt solution at 4°C to remove contaminating somite tissue, the crest material was placed on semisolid culture medium in contact with 12-day-old ventral embryonic skin and cultured for 12–24 h at 37°C in a CO₂-enriched environment.

All grafts were cultured for a further three weeks by placing them beneath the testicular tunica of anaesthetised adult male mice (Russell, 1961; Mayer & Fishbane, 1972). After this period, the mice were killed, their testes dissected out and fixed in formol saline, dehydrated and cleared in benzyl alcohol. The pigmentation of hairs produced by successful grafts was examined at both macroscopic and microscopic levels.

Graft tissue combinations

a) 14-day recombinant skin grafts were constructed as follows:— (i) albino (either *aa cc* or *bb cc*) and pink-eyed dilution (*pp*) skins were separated into their component tissues (i.e. dermis and epidermis) and reconstructed to check the experimental procedure; (ii) reciprocal recombinant grafts between agouti pink-eyed dilution (*AA pp*) and non-agouti wild-type (*aa PP*) skins were constructed as positive controls since agouti gene expression is known to be mediated through the dermis (Mayer & Fishbane, 1972; Poole, 1974; 1975); and (iii) reciprocal recombinant grafts between albino (either *aa cc* or *bb cc*) and pink-eyed dilution (*pp*) skin were constructed to determine whether gene expression is mediated through the dermal tissue environment.

b) 13-day reciprocal recombinant skin grafts were constructed from pink-eyed dilution and homozygous sash skin to test for dermal or epidermal expression of the wild-type allele at the *p* locus and its influence on the melanocyte at the same developmental stage. The melanocytes should be *pp* in both combinations as 13-day dermal melanocytes are capable of migrating into an epidermis which is deficient in its own indigenous melanocyte population (Mayer, 1973a). (i) pink-eyed dilution (*pp*) dermis was combined with sash (*W^{sh}W^{sh}*) epidermis to test epidermal

involvement in melanocyte gene expression; and (ii) pink-eyed dilution (*pp*) epidermis was combined with sash ($W^{sh}W^{sh}$) dermis to test dermal tissue involvement.

c) Neural crest material from 9-day-old pink-eyed dilution (*pp*) embryos was combined with 12-day-old albino [either *bb cc* or *a+ b+ cc* (F_1 JU/FaCt \times BALB/c)] or homozygous sash ($W^{sh}W^{sh}$) skin to test the effect of the intact skin on melanocyte gene expression.

RESULTS

A) 14-day recombinant skin study

Thirty-five of the fifty-eight 14-day recombinant grafts implanted were successfully recovered with epidermal differentiation and twenty-two showed full hair growth (Table 1).

The hair pigmentation found in the control grafts between dermis and epidermis of the same genotype (i.e. *aa cc//aa cc*, *bb cc//bb cc* and *pp//pp*) demonstrates that the manipulation of the tissue does not in itself affect melanocyte gene expression. Albino (either *aa cc* or *bb cc*) recombinants produced exclusively non-pigmented hairs, whereas the pink-eyed dilution (*pp*) recombinants produced hairs with the typical yellow agouti phenotype of *AA pp* mice (Table 1).

In the additional series of positive control grafts, constructed between non-agouti (*aa*) and agouti pink-eyed dilution (*pp*) 14-day embryonic skin (Table 1), no recombinant *pp//aa** grafts were recovered, but all five of the reciprocal grafts (*aa//pp*) were recovered with full hair growth. Hairs produced by these grafts displayed the agouti (i.e. a black hair with a sub-apical yellow band) pigmentation phenotype reflecting the agouti genotype of the dermis rather than the non-agouti genotype of the epidermis and melanocytes confirming that the dermal influence of *A* can be detected.

Absence of detectable pigmentation in the hairs produced by the 14-day *aa cc* or *bb cc//pp* experimental grafts demonstrates that melanogenically active melanocytes do not migrate from the dermis to populate the follicles of the epidermis at this time. In the reciprocal grafts (i.e. *pp//aa cc* or *bb cc*) hair pigmentation was yellow agouti indicative of the agouti pink-eyed dilution genotype of the melanocytes and epidermis irrespective of the agouti (*aa* or *AA*) genotype of the dermis (Table 1) and demonstrating lack of dermal influence of the wild-type *p* allele.

B) 13-day reciprocal recombinant skin study

The results obtained in the 13-day reciprocal recombinant graft study between homozygous sash ($W^{sh}W^{sh}$) and pink-eyed dilution (*pp*) skins are presented in Table 2. Hair pigmentation produced by the *pp//W^{sh}W^{sh}* grafts was typical of the agouti pink-eyed dilution genotype of the melanocytes. This result shows that there is no dermal influence from the wild-type *P* allele. In the reciprocal experiment (i.e. $W^{sh}W^{sh}//pp$), where the melanocytes should arise from the residual population

* epidermal genotype//dermal genotype.

Table 1. 14-day recombinant skin study to test dermal involvement in pigment gene expression at the *p* locus.

Genotype*		Number of grafts			Hair pigmentation phenotype
Epidermis (i.e. melanocyte)	Dermis (i.e. environment)	Implanted	Recovered	Hair growth	
<i>aa cc</i>	<i>aa cc</i>	11	11	3	white
<i>bb cc</i>	<i>bb cc</i>	1	1	1	white
<i>pp</i>	<i>pp</i>	2	1	1	yellow agouti†
<i>pp</i>	<i>aa</i>	1	0	0	—
<i>aa</i>	<i>pp</i>	5	5	5	agouti
<i>aa cc</i>	<i>pp</i>	8	4	3	white
<i>bb cc</i>	<i>pp</i>	10	7	5	white
<i>pp</i>	<i>aa cc</i>	10	2	2	yellow agouti†
<i>pp</i>	<i>bb cc</i>	10	4	2	yellow agouti†

38% of the grafts implanted were successfully recovered with full hair growth.

* Other pigment loci not shown carry the wild-type allele (e.g. *pp* is *AA BB p C/p C*).

† A predominantly yellow hair with poorly developed black (eumelanin) pigmentation typical of the *p* phenotypes.

Table 2. 13-day reciprocal recombinant skin study to test both dermal and epidermal involvement in pigment gene expression.

Genotype*		Grafts		Hair pigmentation phenotype
Epidermis	Dermis	Implanted	Recovered	
<i>pp</i> †	<i>W^{sh} W^{sh}‡</i>	4	1	yellow agouti§
<i>W^{sh} W^{sh}‡</i>	<i>pp</i> †	6	2	yellow agouti§

* Other pigment loci not shown carry the wild-type allele except for ‡.
† Origin of melanocytes.
‡ Homozygous angora (*go*) buff (*bf*).
§ Predominantly yellow-looking hairs typical of the *p* phenotype.

of the dermis, the pigment phenotype of the hair was also typical of the agouti pink-eyed dilution phenotype. This result suggests firstly that the follicular environment provided by the sash epidermis is capable of supporting melanocyte differentiation and secondly that gene expression at the *p* locus is not mediated through the genotype of the epidermal tissue environment.

C) 9-day neural crest study

Eleven well-developed grafts with full hair growth from 9-day neural crest material and 12-day embryonic skin combinations were recovered (Table 3). One graft contained pigmented hairs, the pigmentation was indicative of the melanocyte genotype arising from the neural crest (i.e. *pp*). Absence of pigmentation in the other grafts was probably due to a failure in survival of the neural crest material or its possible loss during the transfer into the testes. Since host skin from two genetically non-pigmented sources (i.e. albino and sash) produced grafts with non-pigmented hairs it is unlikely that they would both suppress pigment production by the invading *p* melanocytes.

Table 3. Results of combined grafts of 8- to 9-day neural crest with 12-day embryonic skin.

Genotype		Grafts recovered*	Hair pigmentation phenotype
Neural crest (i.e. melanocytes)	Skin (i.e. environment)		
<i>pp</i>	<i>aa cc × bb cc</i>	7	white
<i>pp</i>	<i>bb cc</i>	1	yellow agouti†
<i>pp</i>	<i>W^{sh}W^{sh}</i>	3	white

* i.e. grafts with well-developed hairs.
† Predominantly yellow-looking hairs typical of the *p* phenotype.

DISCUSSION

Melanoblasts migrate from the neural crest between the 8th and 12th day of gestation (Rawles, 1947) entering the epidermal component of the skin via the dermis by the 13th day (Mayer, 1973a). The 14-day recombinant skin grafts test for any interaction between melanocytes (by then indigenous to the epidermis) and the cells of the dermal tissue environment. The agouti pink-eyed dilution phenotype (Table 1) shown by the hairs in the *pp/cc* recombinant grafts demonstrates a lack of dermal influence. The normal agouti phenotype of the *aa/pp* recombinants both confirms the dermal influence of the agouti locus and a lack of dermal influence of the *p* locus. The 13-day *pp/W^{sh}W^{sh}* recombinant graft (Table 2), which produced hairs with the yellow agouti phenotype typical of *pp*, also confirms the absence of dermal mediation by genes at the *p* locus. A lack of epidermal influence of genes at the *p* locus was also confirmed by the *W^{sh}W^{sh}//pp* grafts which produced yellow agouti hairs typical of the *pp* melanocytes derived from the dermal component of the recombinant skin. Thus it can be concluded that gene expression at the *p* locus is melanocyte autonomous. The pink-eyed phenotype of the pigmented graft recovered in the neural crest study (Table 3) also supports this conclusion.

These results are experimental confirmation that gene expression at the *p* locus is pigment cell autonomous as deduced by Silvers (1958a) from indirect evidence. Sidman & Pearlstein's (1965) demonstration of an increase in pigmentation in retinal melanocytes taken from eyes of *p* homozygotes cultured *in vitro* on tyrosine-supplemented media could be interpreted as evidence for an environmental influence of genes at the *p* locus, but this increase in pigmentation was:— (i) independent of both RNA transcription and translation; and (ii) dependent upon a tyrosine supplement suggesting an abnormality in tyrosine metabolism within the cell. Other evidence in support of the autonomous expression of genes at the *p* locus is provided by the pink-eyed unstable (*p^{un}*) allele. This particular mutant displays a tendency to revert spontaneously giving rise to pigment mosaics (Wolfe, 1963). The amount of full pigmentation in the coat and eyes is thought to reflect the stage during development when the original reversion occurred (Melvold, 1971). Reversion at later stages may occur (Searle, 1978b) giving rise to small clones of fully pigmented cells within the eyes and individual hair follicles (Searle, 1977). If expression of the *p* locus were mediated by the local tissue environment then small revertant clones would not be detected as pigment production would be suppressed by the dominant influence of the surrounding, non-revertant, tissue. Mosaics in which a large proportion of the coat contains full pigmentation (high-grade mosaics) display the broad banding pattern typical of chimaeras constructed between embryos with different pigment genes whose expression is cell autonomous (e.g. *cc ↔ CC*) and not the fine banding pattern found in chimaeras constructed from embryos with environmentally mediated pigment gene expression (e.g. *AA ↔ aa*) as demonstrated by Mintz (1974).

The agouti pigmentation phenotype of hairs produced by the 14-day *aa/pp*

recombinant grafts is in agreement with previous reports of the dermis as the site of gene expression at the agouti locus (Mayer & Fishbane, 1972; Poole, 1975). However, the agouti phenotype of hairs produced by the 14-day $pp//aa$ cc recombinant grafts is inconsistent with these reports. The reason for this conflict is unclear but it may reflect some epistatic influence of the p gene on other pigment loci. Expression of the light (B^h) phenotype is known to be modified in mice heterozygous for p (Silvers, 1979). Similarly, mice homozygous for ruby-2 ($ru-2$) and heterozygous for p have lighter coats than litter mates which do not carry the p gene (Stephenson, unpublished information). Yellow ($A^y a$) epidermis is not overruled by non-agouti (aa) dermis (Poole, 1974; 1975). Since pink-eyed dilution melanocytes are capable of normal phaeomelanin but only poor eumelanin production, it is possible that the AA pp genotype gives a genetical weighting in the epidermis and the melanocytes in favour of phaeomelanin production so that aa dermis is unable to prevent phaeomelanin production by the AA pp melanocytes.

The agouti pink-eyed dilution phenotype of hairs produced by the $W^{sh}W^{sh}//pp$ 13-day recombinant grafts provides additional information on the expression of the W^{sh} gene. It demonstrates that the W^{sh} epidermis, in common with other alleles at the W locus (i.e. W , dominant spotting; W^v , viable dominant spotting), is:— (i) capable of supporting both melanocyte differentiation and subsequent pigment production; and (ii) devoid of, or at least deficient in, its own population of melanocytes. If there are any W^{sh} melanocytes present they are apparently excluded from populating the developing hair follicles by melanocytes that do not carry the defective W gene, as suggested by Gordon (1977) and Mayer (1979) for the other alleles. These experiments have demonstrated the value of W^{sh} homozygotes in experimental systems for testing the site of gene expression of loci controlling pigmentation. The ability to breed from homozygotes removes the problem of identifying embryonic genotype encountered by Mayer (1973*b*). Absence of W^{sh} melanocytes allows the use of a 13-day reciprocal recombinant system to test separately for both dermal and epidermal influence of genes affecting pigmentation.

DAS acknowledges the support of an SERC case studentship held between the Zoology Department, Reading and the MRC Radiobiology Unit, Harwell. We are grateful to Dr T. Cooper for demonstrating the surgical techniques and to Mrs P. Crocker for typing the manuscript.

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(Accepted 7 January 1985)