The site of action of the naked locus (N) in the mouse as determined by dermal-epidermal recombinations

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SUMMARY

The dermal-epidermal recombination technique was used to determine the site of action of the naked (N) locus in the skin of the mouse. The skin of athymic (nude) mice was used as a host site for growth of recombined epidermis and dermis from 13- and 14-day N/+ and +/+ embryos. Grafts that contained mutant epidermis lost their hair by 26 days after grafting (at the end of the first hair cycle) and again after 47 days (at the end of the second hair cycle); grafts that contained normal epidermis retained hair throughout the experiment. It was concluded that the N locus acts in the epidermis.

INTRODUCTION

The naked gene (N), first described by Lebedinsky & Dauvart (1927), is a semi-dominant gene affecting the hair coat of the mouse. In heterozygotes N causes a slight delay in the time of eruption of the first coat (Fraser & Nay, 1955), shedding by breakage of the first coat between 10 and 20 days of age and cyclic regeneration and loss of subsequent hair coats at about monthly intervals (David, 1932). The homozygotes generally die within the first week of life, and in those that do survive, hair development is markedly abnormal with invariable failure of eruption of the first hair coat (David, 1932). In order to determine the mode of action of N during the morphogenesis of hair, knowledge of the primary tissue of activity of the gene is necessary. David (1934) who carried out reciprocal skin transplants and parabiotic unions between N/+ and +/+ mice, concluded that N affected the skin directly rather than through an endocrine abnormality. The purpose of this investigation is to determine whether N has its primary activity in the dermal or the epidermal component of the skin.

The development of a method of splitting skin into epidermis and dermis

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with a dilute solution of trypsin (Medawar, 1941; Szabó, 1955) opened the way for a wealth of experiments designed to elucidate the interactions between the dermis and epidermis during the development of skin appendages. By means of this technique, it has been established that the dermis determines the position of hair follicles, the type of appendage formed and its gross morphology (Kollar, 1972; Dhouailly, 1977). The morphological details (of feathers at least) and the chemical composition of the appendages are determined by the epidermis (Sengel & Dhouailly, 1977). During the cycle of growth (anagen) and rest (telogen) of the hair follicle, the dermal papilla is essential for the initiation of hair growth at the beginning of each hair cycle (Oliver, 1971). The epidermal-dermal recombination technique has also been used to determine the site of action of several mutant genes affecting the hair coat in the mouse, namely hairless (Billingham & Silvers, 1973), downless (Sofaer, 1973, 1974), tabby (Sofaer, 1974; Mayer & Green, 1978), fuzzy (Mayer, Mittelberger & Green, 1974), ichthyosis (Green, Alpert & Mayer, 1974), depilated (Mayer, Kleiman & Green, 1976) and crinkled (Mayer, Miller & Green, 1977). Of the seven mutants investigated, six affect details of hair structure and have activity in the epidermis. The seventh, hairless (hr), which affects the orderly progress of catagen and the eruption of second and subsequent hair cycles (David, 1932; Orwin, Chase & Silver, 1967), was found to act in the dermis (Billingham & Silvers, 1973).

In these experiments recombination of skins from embryos of 14 days or older, or from newborn mice (hr) were used and first cycle hairs or follicles were examined for abnormalities. By 14 days gestation hair follicles are visible on the body skin of the mouse embryo (Gruneberg, 1943). By forming recombinants at this time, an earlier transient effect of mutant dermis on the epidermis could have been missed (Mayer et al. 1974). An early effect of mutant dermis could be picked up by forming recombinants at an earlier time or by allowing the recombined skins to grow a second hair cycle, since many, if not all, of the interactions which occur during the development of the hair follicle in the embryo are probably repeated at the beginning of each hair cycle (Oliver, 1971). Therefore, to try and ensure that an effect on the dermis was not being missed we have recombined skin from 13-day embryos as well as from 14-day embryos, and some recombined grafts from 13-day embryos were allowed to develop a second hair cycle.

Green et al. (1974), who transplanted skin from normal and naked (N/+ ) 14-day embryos under the testis capsule of histocompatible adult males, could find no differences between the grafts 14 days after implantation. They concluded that this graft site was unsuitable for studying mutants which affect hair shedding or which have only minor effects on hair morphology. In the experiments described below the skin of the athymic (nude) mouse was used as the graft site for the dermal-epidermal recombinants. By allowing the grafts to grow for 26 days, or for 47 days for the second hair cycle, it was possible to
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observe whether telogen hairs were retained on the graft or shed as in N/+ mice. The results suggest that N acts in the epidermis.

MATERIALS AND METHODS

The naked stock used in these experiments was a random-bred coloured stock formed by crossing an inbred naked stock from the Jackson Laboratory, Maine, to a local coloured strain. Subsequent to this initial cross, the stock was selected on the basis of the ability of the mice to rear their N/N offspring. The nude mice (nu/nu) used as hosts were from a random-bred albino stock formed initially by crossing Re +/+ nu males from the Institute of Animal Genetics, Edinburgh to Quackenbush females. Both the naked and the nude stocks were maintained under conventional conditions.

N/+ embryos were obtained by pairing +/+ females with N/N males; +/+ embryos were obtained by pairing +/+ females with +/+ males. The morning on which a vaginal plug was found was considered day 0 of pregnancy. After 13 or 14 days, the females were killed by cervical dislocation and the uterus removed. The embryos were dissected from the uterus and placed in Tyrode’s solution. Pieces of skin approximately 3 × 2 mm were cut from both flanks of each embryo with iridectomy scissors. Muscle adhering to the dermis was removed with forceps, and the skin pieces were transferred to a dish of Tyrode’s solution containing 1 % trypsin (Difco 1:250). This dish was held at 4 °C until the epidermis began to loosen at the edges (about 1½ h for 13-day skin; about 4 h for 14-day skin). The skin pieces were then transferred to a dish containing 20 % foetal calf serum in Tyrode’s solution to inhibit further action of the trypsin. This dish was placed in an ice-box and the dermal and epidermal components were separated with watchmakers forceps under a dissecting microscope using a cold-light source (Volpi Intralux fibre optic light). Following separation the skin components were recombined on an agar-based culture medium (Eagle’s basal medium containing foetal calf serum (10%), agar (1·5%), streptomycin (100 μg/ml), penicillin (100 units/ml) and fungizone (5 μg/ml)). With a small spatula, the dermis was transferred from the Tyrode’s solution to the agar plate and several drops of cold 20 % foetal calf serum were added. The epidermis was then placed beside the dermis and drawn over the top of the dermis. The recombined skins were incubated overnight at 37·5 °C in an atmosphere of 5 % CO₂. Unseparated skin pieces from +/+ and N/+ embryos were also incubated on agar overnight.

Healthy nude mice of about 5 weeks of age were used as hosts. Two grafts were placed on each host, one on each side. When the host had been anaesthetized with ether, the graft bed was prepared by removing a piece of skin about 5 × 4 mm with curved scissors from above the lateral thoracic blood vessel. The recombined embryonic skin was transferred to the graft bed on a small spatula. The graft was covered with a 5 mm² of dialysis tubing (sterilized in 70 %
alcohol, rinsed in Tyrode's solution), and with a spot bandaid (Johnson and Johnson Pty Ltd). The host was then turned over and another graft placed on the other side. When both grafts were in place, the host was bandaged first with 1.25 cm wide Leukopore tape (Beiersdorf) and then with 2.5 cm wide Leukoplast tape (Beiersdorf). The Leukoplast was joined with its sticky surfaces together on the dorsal surface of the host and stapled close to the body of the animal. When the mice had regained consciousness, they were placed in a cage with other grafted animals. The cages were kept in a warm atmosphere (c. 25 °C).

Bandages were removed from the host mice 6 days after grafting. At this time, and at intervals of 5–7 days thereafter, the grafts were examined under a dissecting microscope for signs of hair development, hair growth and shedding. The observer making these observations was unaware of the genotypes of the graft components. After 26 or 47 days on the nude hosts, the grafts were removed and fixed in formol saline.

RESULTS

Graft ages have been expressed as the time interval in days since conception of the donor mouse (cf. Claxton, 1966). When time of conception is used as the starting point for calculating ages, eruption of the first hair coat occurs at about 24 days (i.e. 5 days after birth), breakage of the first coat hairs in N/ + mice at about 40 days, eruption of the second hair coat at about 47 days and breakage of the second hair coat in N/ + mice at about 61 days. Because N/ + skin is bare at 40 days of age and again at 61 days, it was possible to classify grafts of these ages into those which were phenotypically +/+ and those which were phenotypically N/+.+

Grafts were rejected from the experimental results if the host died before the graft reached 40 days of age (or before 61 days for second hair cycle grafts) or if hair failed to erupt by 28 days either as a result of degeneration of the graft itself or as a result of overgrowth by the skin of the host. Of the 98 nude mice grafted, 86 survived for 40 days donor age, and of the 165 skin pieces grafted to these surviving mice (28 unseparated, 137 recombined) 98 were suitable for inclusion in the experimental results (16 unseparated, 82 recombined). Of the 19 hosts kept to observe the second hair cycle on the grafted skin, 17 survived for 61 days donor age, and of the 22 recombined skin grafts on these surviving hosts, 21 were suitable for inclusion in the experimental results. One recombined graft (N/ + epidermis and N/ + dermis) which grew first cycle hairs failed to grow a second coat. To test the completeness of the separation of the dermis and epidermis, 8 pieces of dermis alone and 13 pieces of epidermis alone were grafted to nude hosts. None of these grafts showed any sign of hair growth.
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Grafts of unseparated skins

In +/+ mice guard hairs erupt on the flank between 22 and 24 days post conception and coat hairs between 25 and 27 days. The tips of these hairs are very rarely broken. In N/+ mice eruption of the first coat is slightly delayed (guard hairs 23–25 days; coat hairs, 26–28 days) and the hair tips are often bent or broken. By 31 to 32 days the hairs reach full length in both genotypes, but by 40 days when the hair follicles are in telogen, most of the N/+ hairs break off close to the skin surface (David, 1932; Fraser & Nay, 1955).

In order to determine whether skin grafts from +/+ and N/+ donors behave in a manner similar to skin left in situ samples of unseparated skins from +/+ and N/+ 14-day-old embryos were grown on agar overnight and grafted to the nude hosts the following day. Figures 1, 2, 3 and 4 illustrate the changes observed in an unseparated skin graft from a 14-day +/+ donor. When the bandage was removed from the host mouse at 21 days donor age, pigment spots marking the site of the developing follicles were visible on the graft. By 24 days the graft bed had thickened and a few hairs had erupted. The tips of these hairs were straight and undamaged. By 28 days the hairs had grown to about 3 mm in length. By 33 days, the hair on the graft had reached full length and it was apparent that all four hair types were represented on the graft. By 40 days when the hair follicles were in telogen, the graft still retained a full crop of hair. Figures 5, 6, 7 and 8 illustrate the changes observed in a whole skin graft from a 14-day N/+ donor. In these grafts, hair tended to erupt later (24–28 days) than in grafts from +/+ donors. The tips of the erupting hairs were often bent or broken like those of N/+ mice and the grafts were bare by 40 days. Thus, the behaviour of both +/+ and N/+ grafts was very similar to that of skin left on the young mouse.

Recombinations of epidermis and dermis from 13- and 14-day +/+ and N/+ embryos grown for 26 days on nude hosts

Four types of skin recombinations were made: +/+ epidermis and +/+ dermis, +/+ epidermis and N/+ dermis, N/+ epidermis and +/+ dermis, and N/+ epidermis and N/+ dermis. Table 1 shows the results of the four epidermal-dermal recombination types. Recombinants with +/+ epidermus and +/+ or N/+ dermis possessed hair at 40 days donor age (with one exception), recombinants with N/+ epidermis and +/+ or N/+ dermis were without hair at 40 days donor age. Thus it is the genotype of the epidermis which determined the type of hair produced in the recombined skin grafts. Figures 9, 10 show the appearance of a graft of +/+ epidermis and N/+ dermis at 33 days and 40 days donor age. Figures 11, 12 show the appearance of a graft of N/+ epidermis and +/+ dermis at 33 days and 40 days donor age. One 14-day graft with +/+ epidermis and N/+ dermis lost most of its hair by 40 days. This graft may have contained some N/+ epidermis as a result.
Table 1. Results of epidermal-dermal recombinations between 13- and 14-day +/+ and N/+ embryonic mouse skin grown for 26 days (40 days donor age) on the nude mouse

<table>
<thead>
<tr>
<th>Recombination</th>
<th>Age of donor (days gestation)</th>
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<th></th>
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<tbody>
<tr>
<td></td>
<td>13 days</td>
<td>14 days</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Grafts with hair</td>
<td>Grafts without hair</td>
<td>Total</td>
</tr>
<tr>
<td></td>
<td>13 days</td>
<td>14 days</td>
<td></td>
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<tr>
<td></td>
<td>Grafts with hair</td>
<td>Grafts without hair</td>
<td>Total</td>
</tr>
<tr>
<td>+/+</td>
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<td>0</td>
<td>7</td>
</tr>
<tr>
<td>+/+</td>
<td>9</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>N/+</td>
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<td>12</td>
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</tr>
<tr>
<td>N/+</td>
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<td>16</td>
<td>16</td>
</tr>
<tr>
<td>N/+</td>
<td>6</td>
<td>7</td>
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</tr>
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</table>

of incomplete separation of the N/+ embryonic skin. This could result in the growth of N/+ hairs on the graft. Those hairs that were still on the graft at 40 days were mainly zig-zags and probably originated from the +/+ epidermis since N/+ grafts never had zig-zags at 40 days.

Recombinations of dermis and epidermis from 13-day +/+ and N/+ embryos grown for 47 days on nude hosts

Some 13-day recombined skin grafts which grew first cycle hairs successfully were retained until the second cycle of hairs had entered telogen at about 61 days. This enabled us to observe whether mutant dermis had any effect.
Grafts of recombined 14 day embryonic skins grown on nude mice for 26 days

Fig. 9. ++ epidermis, N/+ dermis at 33 days donor age (lateral view)
Fig. 10. ++ epidermis, N/+ dermis at 40 days donor age (lateral view)
Fig. 11. N/+ epidermis, ++ dermis at 33 days donor age (lateral view)
Fig. 12. N/+ epidermis, ++ dermis at 40 days donor age (vertical view)

on the morphology or the shedding of second cycle hairs. Table 2 shows the genotype and number of grafts with and without hair at 61 days. The first cycle hairs were shaved off at 40 days from grafts with ++ epidermis. The second cycle hairs erupted between 47 days and 53 days. Grafts with ++ epidermis retained their hair until the end of the experiment. In grafts with N/+ epidermis, the second cycle hairs often had bent and broken tips like first cycle hairs and when the hair follicles entered telogen at 61 days, the second cycle hairs were lost. Thus the genotype of the epidermis determined the type of hair produced during the second cycle in the recombined skin grafts.

DISCUSSION

The results of these experiments strongly suggest that the loss of hair in N/+ mice is due to the activity of N in the epidermis. Presence of the mutant
Table 2. Results of epidermal-dermal recombinations between 13-day +/+ and N/+ embryonic mouse skin grown for 47 days (61 days donor age) on the nude mouse

<table>
<thead>
<tr>
<th>Recombination epidermis dermis</th>
<th>Grafts with hair</th>
<th>Grafts without hair</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>+/+</td>
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<td>6</td>
</tr>
<tr>
<td>N/+</td>
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</tr>
<tr>
<td>N/+</td>
<td>0</td>
<td>5</td>
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</tr>
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</table>

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gene in the epidermal component of recombinated skin grafts consistently resulted in loss of hair from the grafts after 26 days and again after 47 days growth on nude hosts. With one possible exception no effect of the dermis was evident even in the youngest recombinations (13-day embryonic skin) or in grafts which grew a second cycle of hairs. This result is consistent with the site of activity found for other mutations which affect hair structure. It is in contrast to hairless which, like naked, causes shedding of the first hair coat between 10 and 20 days, but which has activity in the dermis (Billingham & Silvers, 1973). In hairless (hr/hr) mice the first coat is normal, but the hair club is improperly formed and shedding is caused by the hair falling out, while in N/+ mice the hair breaks close to the skin when fully grown (David, 1932). The cause of shedding in these two mutants is thus quite different.

N/+ mice produce hair that is deficient in the high-glycine, high-tyrosine (HG) proteins of keratin (Tenenhouse, Gold, Kachra & Fraser, 1974; Tenenhouse & Gold, 1976) which are important in the stabilization of the matrix structure of the hair cortex (Bendit & Gillespie, 1978). Thus deficiency of these proteins could cause breakage of hairs. However, it is unlikely that N is directly involved in the synthesis of HG proteins because the characteristics of homozygous naked mice suggest that N affects cellular processes other than those involved in the formation of hair. N/N mice usually die within the first week after birth and those which do survive grow more slowly than +/+ or N/+ mice (Lebedinsky & Dauvart, 1927; Ebbenhorst Tengbergen, 1939). Very short fuzzy hairs do erupt but the cuticle is indistinct or seems to be entirely absent (David, 1932). The hair follicle bulbs of N/N mice are smaller than those of +/+ or N/+ mice and the inner root sheath cuticle and hair cuticle often fail to differentiate (David, 1932). These observations suggest that, in
double dose, \( N \) affects cell numbers as well as the quantity of protein produced. These experiments demonstrate the suitability of the skin of the nude mouse as a host site for the growth of recombined embryonic skin. Due to the ability of these mice to accept allografts (Pennycuik, 1971), it is not necessary to use inbred mutant stocks. The skin provides a more natural environment than the testis capsule, for example, for the development of hair in skin grafts since the hair erupts into air rather than fluid and is not forced to lengthen by abnormal downgrowth of follicles into the soft testicular tissue (Mayer et al. 1976). Also because of the location of the grafts on the skin surface, it is possible to observe the grafts continuously after the removal of the bandages from the host mice.

The execution of these experiments would not have been possible without the skilled technical assistance of Mrs Barbara Frangleton and Mr Vincent Tongue, or the advice of Dr Helen Briscoe who kindly showed us her technique for applying grafts to nude mice.

REFERENCES


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