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# Eyelid development, fusion and subsequent reopening in the mouse

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## ABSTRACT

The process of eyelid development was studied in the mouse. The critical events occur between about 15.5 d postcoitum (p.c.) and 12 d after birth, and were studied by conventional histology and by scanning electron microscopy. At about 15.5 d p.c. the cornea of the eye is clearly visible with the primitive eyelids being represented by protruding ridges of epithelium at its periphery. Over the next 24 h, eyelid development proceeds to the stage when the cornea is completely covered by the fused eyelids. Periderm cells stream in to fill the gap between the developing eyelids. Their proliferative activity is such that they produce a cellular excrescence on the outer surface of the line of fusion of the eyelids. This excrescence had almost disappeared by about 17.5 d p.c. Keratinisation is first evident at this stage on the surface of the eyelids and passes continuously from one eyelid to the other. Evidence of epidermal differentiation is more clearly seen in the newborn, where a distinctive stratum granulosum now occupies about one third of its entire thickness. Within the subjacent dermis, hair follicles are differentiating. By about 5 d after birth, a thick layer of keratin extends without interruption across the junctional region. While a noticeable surface indentation overlies the latter, a similar depression is only seen on the conjunctival surface by about 10 d after birth. Keratinisation is also observed to extend in from the epidermal surface to involve the entire region between the 2 eyelids at about this time. Numerous mature hair follicles are also present within the dermis at this stage, as well as differentiated muscle fibres of orbicularis oculi. By about 12 d after birth, squames of keratin are located between the 2 eyelids, and eyelid opening occurs rapidly thereafter. While the sequence of events in the mouse is similar to that described in the human, the histological events associated with the closure and subsequent reopening of the eyelids have not previously been described in detail in any species.
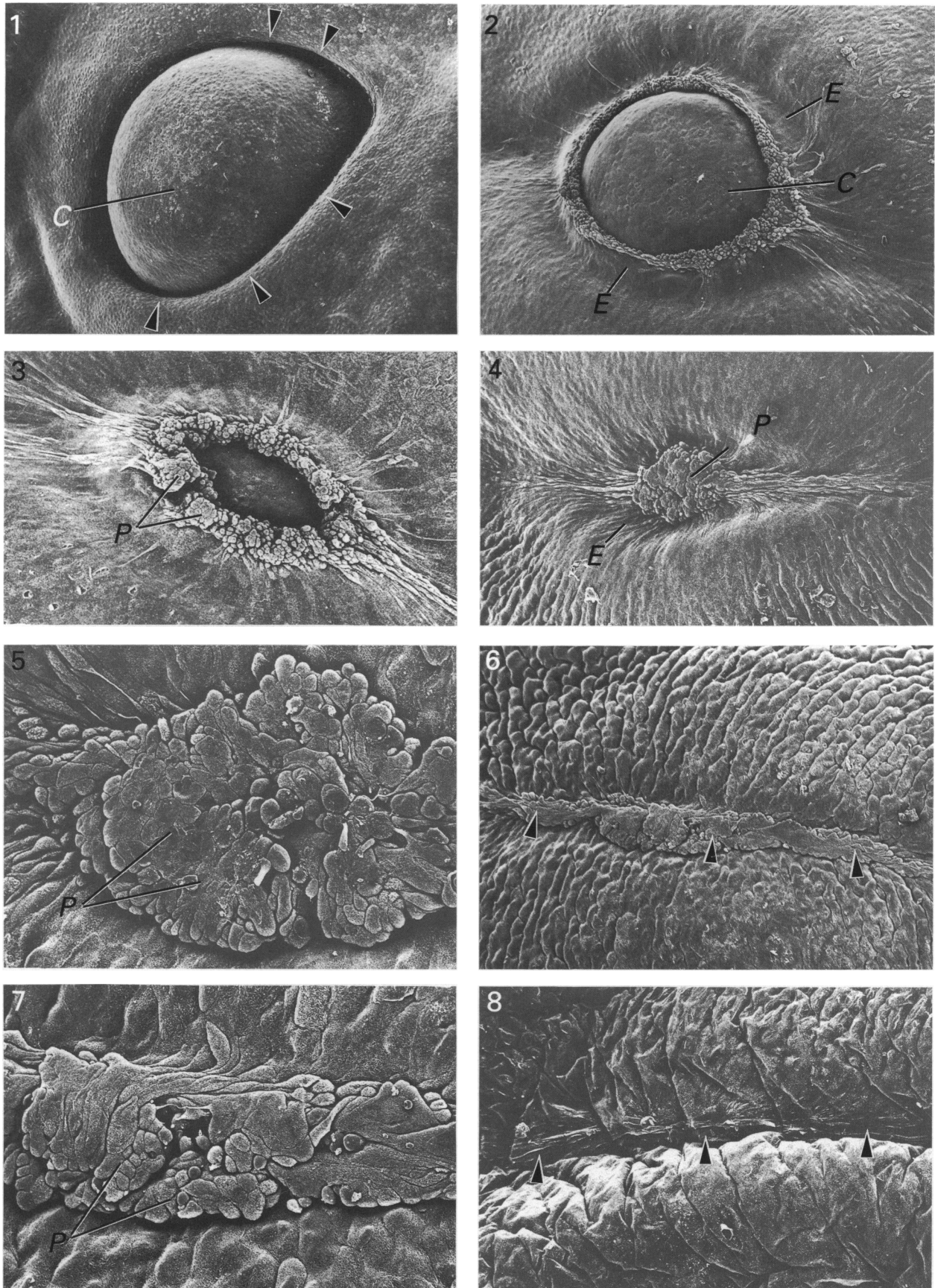
## INTRODUCTION

Eyelid development, fusion and subsequent reopening were first described in the rat by Addison & How (1921). They found that in a 17 d fetus the primitive eyelids had the form of protruding ridges which were just visible around the margins of the developing eye. However, subsequent eyelid development was apparently so rapid that by d 18 of gestation the eyes were completely covered by the fused eyelids. At birth, gestational age d 22, the eyelids were tightly closed and remained so until 14 d after birth, when eyelid opening finally occurred.

Eyelid development, closure and subsequent reopening is a feature common to all mammals. Whether a particular animal species is born with its eyelids open or closed is believed to be determined by the stage of development of the animal at the time of birth. In human embryos, for example, the eyelids start to form at about 40–45 d of prenatal life with complete fusion occurring approximately 15 d later (Pearson, 1980). The eyelids remain closed thereafter until the seventh month of fetal life when they reopen (Hamilton & Mossman, 1972).

In their work on the prenatal development of the mouse eye, Pei & Rhodin (1970) made passing observations on the development of the eyelids. They found that formation of the eyelids in the mouse began at approximately 13–14 d of gestation with fusion occurring sometime during the subsequent 2 d. They did not, however, observe events after birth and therefore did not view the process of eyelid dys-

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Figures 1 to 7 are scanning electron micrographs from the period gestational age 15.5 to 16.5 d p.c.

Fig. 1. Early in this period the cornea (*C*) can be clearly seen within the margins of the developing eyelids (arrowheads) × 100.

junction. Harris & McLeod (1982) carried out a scanning electron microscopic study on eyelid growth and fusion in embryonic/fetal mice. They confirmed many of the observations of Addison & How (1921) and of Pei & Rhodin (1970), but did not follow the process through to the stage of eyelid reopening.

Whereas other studies have used light microscopy to investigate eyelid development in the rat (Addison & How, 1921) and mouse (Pei & Rhodin 1970), and the scanning electron microscope to observe the external features of eyelid development in the mouse (Harris & McLeod, 1982), and then only as far as eyelid fusion, this study consists of both a light and scanning electron microscopic investigation of eyelid development in the mouse, covering the period from eyelid formation, apposition and fusion, through to their eventual reopening at just under 2 wk after birth.

MATERIALS AND METHODS

Spontaneously cycling female C57BL × CBA F1 hybrid mice were mated with F1 hybrid males and isolated on the morning of finding a vaginal plug. This was designated the first day of pregnancy, or 0.5 d postcoitum (d p.c.). Individual females were killed by cervical dislocation at various times between 14.5 d p.c. and full term (approximately 20 d p.c.), and their embryos isolated into phosphate buffered saline. The embryos were dissected free of their extraembryonic membranes and decapitated, and the heads fixed by immersion in 3% glutaraldehyde in 0.1 M phosphate buffer. The heads were then split in the midline in the sagittal plane. One half of each head was processed for scanning electron microscopy (SEM), and the other half embedded in Araldite and sectioned for viewing by conventional light microscopy.

A selection of newborn mice and weanlings isolated at various days between birth and 14 d of age were killed by ether anaesthesia. These were decapitated and the heads processed for analysis by SEM and light microscopy. The material that was processed for SEM

analysis was dehydrated, critical point dried, sputter coated with gold, and then viewed in a JEOL JSM 5200 scanning electron microscope. The material that was to be analysed histologically was postfixed in 1% osmium tetroxide, dehydrated and embedded in Araldite prior to sectioning at 1 μm. The sections were stained with 1% toluidine blue. Between 5 and 8 embryos/weanlings were analysed at each of the developmental stages studied (i.e. from 14.5 d p.c. to 14 d postnatal).

RESULTS

Although pregnant animals of the same gestational age were examined, it soon became apparent that the embryos they contained were at slightly different stages of development. Consequently in the following description of eyelid formation, it is the range of events at a given gestational age that is described.

*Gestational age 15.5 days p.c.*

A scanning electron micrograph of an embryo at this stage of eyelid development is shown in Figure 1, where the cornea of the developing eye can be clearly seen within the protruding ridges of the future eyelids. When viewed in the light microscope (Figs 9, 10), the leading edge of the epithelium is seen to be formed from a loose aggregation of cells growing out from each future lid across the corneal surface. The cells at the leading edges appear to be piled up on the external surface of the lid. Distant from the growing edge, the cells of the epidermis consist of a single basal layer of low columnar cells overlain with 3–4 layers of flattened cells. Mitosis is common in both basal and adjacent cell layers. Periderm cells and their associated nuclei are present on the outer surface of the epidermis. The epidermis, where it is reflected back from the growing edge of the eyelid onto its conjunctival surface, is only 2–3 cells thick and therefore much thinner than that

Fig. 2. As development progresses the epidermis (*E*) of the eyelids appears to be streaming across the corneal surface (*C*) × 150.

Fig. 3. At higher magnification, the ruffled margins of the eyelids observed in Figure 2 are seen to be formed by a proliferation of peridermal cells (*P*) which are overlying the epithelium. × 150.

Fig. 4. Recently fused eyelids showing streaming of the epidermis (*E*) towards the point of fusion. Also clearly seen is the accumulation of peridermal cells (*P*) along the junctional region × 150.

Fig. 5. A high magnification view of the cellular excrescence produced by the peridermal cells (*P*) seen in Figure 4. × 500.

Fig. 6. This is of an established junctional region late in this period of development. The extent of the cellular proliferation along the line of fusion is clearly seen (arrowheads) × 150.

Fig. 7. A high magnification of the junctional region shown in Figure 6. P, peridermal cell proliferation. × 500.

Fig. 8. Newborn. By this time the eyelids are tightly fused along the junctional region (arrowheads) and the cellular proliferation, evident in previous micrographs, is no longer present. × 150.
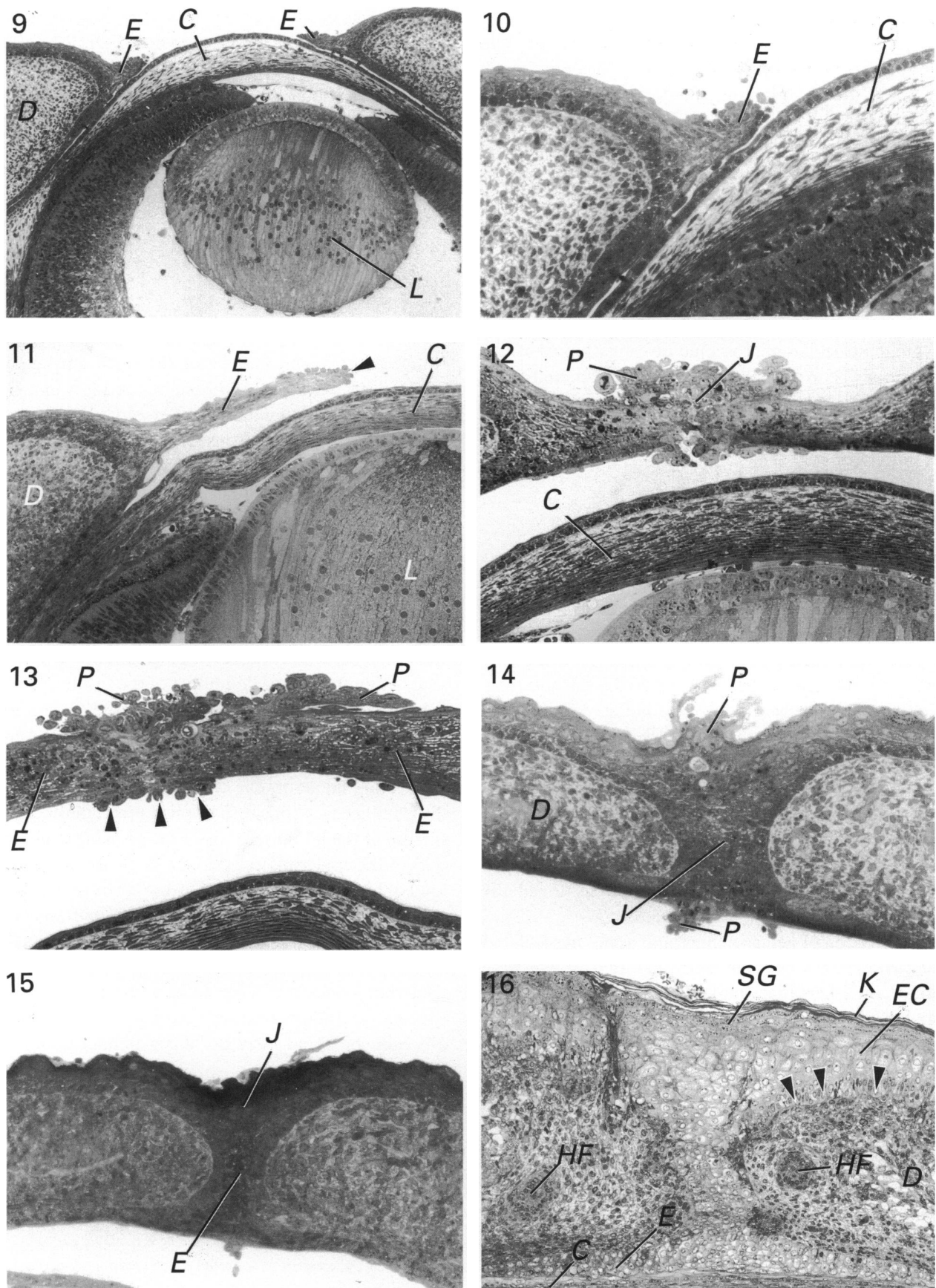
Figures 9 to 13 are light micrographs, gestational age 15.5 d p.c.

Fig. 9. The cornea (*C*) is seen protruding between the developing eyelids. The first signs of the epidermis (*E*) extending across the corneal surface can be seen. *D*, dermis, *L*, lens. × 100.

on the outer surface. The dermis at this stage of eyelid development consists of a loose network of cells and blood vessels.

As development progresses, the epithelium of each eyelid appears to be streaming across the cornea towards each other (Figs 2, 3). The epidermis is thickest where it overlies the dermis at the edge of the eyelid (Fig. 11). Periderm cells are evident on the outer surface of each lid and these appear to be continuous with a less obvious covering of peridermal cells extending a short distance along the conjunctival surface of the eyelids. It is now apparent that it is the periderm which is responsible for the cells which are loosely situated on the surface of the leading edge of the outgrowing epithelium.

Figures 4 and 5 are SEM views of recently apposed eyelids. An analysis of the zone of apposition (Fig. 12), however, reveals that rather than being fused, the junctional zone between the 2 eyelids is formed from a narrow band of loosely apposed cells. Adjacent to the junctional zone, both on the external surface and to a lesser extent on the conjunctival surface of the eyelids, peridermal cells appear to be piling up as if being pushed aside by the coming together of the epithelium of the 2 eyelids. Keratohyalin granules are seen for the first time in the outermost layers of the epidermis distant from the junctional zone.

The dermis of both upper and lower lids appears to be left behind as rapid proliferation of the overlying epithelium takes place. The first signs of muscle formation – representing the future orbicularis oculi – are clearly seen scattered throughout the proximal part of the dermis of both eyelids. The earliest stages of hair follicle development are also present.

Figures 6 and 7 are from an established junctional area, and show the extent of proliferation of cells on the outer surface where the 2 eyelids meet. When a section is taken through this region (Fig. 13), the cellular excrescence is seen to involve both their outer and conjunctival surfaces, although it is much less marked on the conjunctival surface. At this site the cells in the vicinity of the junction are rounded and irregularly arranged.

## Gestational age 16.5 d p.c.

By this time (Fig. 14) the dermis has extended in towards the junctional area, leaving a band of epithelial cells extending from the external surface to the conjunctival surface. The cellular excrescence on the external surface is attached by a stalk-like structure to the line of fusion. The cells pushed out onto the conjunctival surface are, as before, fewer in number than on the outer surface. Distant to the area where the eyelids meet, the epidermis is thrown into numerous folds. Stratification of the epidermis is now evident in this location: the basal layer is low columnar, and superficial to this lie several layers of paler staining cells. The external epidermal surface consists of 2–3 rows of flattened cells which have within them numerous keratohyalin granules. These granules are not evident in the cellular excrescence attached to the line of fusion. The epithelium of the conjunctivum is much thinner than that of the epidermis, does not appear stratified, and does not contain keratohyalin granules. Within the dermis numerous primitive hair follicles are now present, with the cells of the dermis still concentrated near the conjunctival surface.

## Gestational age 17.5 d p.c.

The most obvious feature at this stage of eyelid development is the virtual disappearance of the

Fig. 10. A higher magnification view of the epidermis (*E*) seen in Figure 9 showing that where it passes onto the cornea (*C*) it consists of a loose aggregation of cells. × 250.

Fig. 11. This shows the leading edge (arrowhead) of epidermis (*E*) streaming out across the corneal surface (*C*). The epidermis is thickest where it passes out from the underlying dermis (*D*), *L*, lens. × 160.

Fig. 12. The junctional region (*J*) of recently fused eyelids consists of a loose grouping of cells overlain by peridermal cells (*P*) which appear to be spilling out onto both the internal and external surfaces. *C*, cornea. × 350.

Fig. 13. A tight junction now exists between the fused eyelids with no gaps visible between the epidermis (*E*) of opposing eyelids. The peridermal cells (*P*) are seen extending out across the outer surface from the point of eyelid fusion. A smaller accumulation of cells is seen on the conjunctival surface (arrowheads). × 320.

Fig. 14. Gestational age 16.5 d p.c. The dermis (*D*) has extended in towards the junctional region (*J*) producing a thickening of the eyelids. Periderm cells (*P*) are still present on both surfaces of the eyelids. × 250.

Fig. 15. Gestational age 17.5 days p.c. Further dermal proliferation in towards the junctional region (*J*) has produced a narrowing of the epidermis (*E*) passing between the eyelids. By this time the cellular excrescence has all but disappeared. × 160.

Fig. 16. Newborn, 20 days p.c. The eyelids are now much thicker. The epidermis can be seen to be differentiated into a basal layer of columnar cells (arrowheads), 2 or 3 layers of undifferentiated epidermal cells (*EC*), a stratum granulosum (*SG*) and several outer layers of keratin squames (*K*). On the conjunctival surface, adjacent to the cornea (*C*), the epidermis (*E*) is much thinner and nonkeratinised. Immature hair follicles (*HF*) are present in the dermis (*D*) × 160.

cellular excrescence on the external surface overlying the junctional zone (Fig. 15). The first signs of keratinisation are apparent by now and are evident in the region which bridges the 2 eyelids. Keratohyalin granules are restricted to the most superficial layer of cells immediately underlying the keratin layers and cell division is a commonly observed feature in the basal layer.

The conjunctival epithelium is very much as before, being thinner than the epidermis. Although the cellular excrescence on the external surface had disappeared, a small cellular excrescence on the conjunctival surface of the fused eyelids was often seen (Fig. 15). Apart from early evidence of hair follicle development, which is moderately more advanced in its differentiation than previously observed, the dermis is similar in appearance to that seen at 16.5 days p.c.

### New-born 20 d p.c.

In the new-born mouse (Fig. 16) the junctional epithelium remains thin relative to the overall thickness of the eyelids. The basal layer of cells on the epidermal side is columnar in morphology and remains so as it passes between the 2 lids and onto the conjunctival surface. On the inner aspect of the eyelids just distant to the point of union, the conjunctival epithelium thins from approximately 6–8 cells to about 3–4 cells in thickness. Where the conjunctivum thins, the cells also become much flatter in appearance.

On the epidermal surface there are now several layers of keratin present subjacent to which lies a distinctive stratum granulosum which occupies about one-third of the entire thickness of the epidermis. An SEM of this region (Fig. 8) clearly indicates that the cellular excrescence previously located along the line of union of the 2 lids is no longer evident.

Within the dermis, hair follicles are more numerous and show a greater degree of differentiation than previously. However, as yet, no hair follicles extend from the dermis through the epidermis onto the surface. The first clear signs of tarsal gland development are also present and are located within the dermis close to the margins of the eyelids.

### Postnatal d 5

Figure 17 shows the area of eyelid fusion 5 d after birth. On the external surface keratin extends without interruption across the junctional region. However, there is a noticeable surface depression overlying the latter. The cells of the stratum granulosum underlying this depression are larger and rounder than elsewhere. The epithelium on the conjunctival side of the eyelid junction consists of round cells with relatively unstained nuclei, unlike the smaller more densely stained cells of the conjunctival epithelium distant from the junctional area. The cells of the basal layer are more darkly staining, particularly in the epithelium of the junction, and where this passes round onto the conjunctival surface.

Hair follicles are now numerous in the dermis and some of these contain hair shafts. Because of the density of hair follicles within the dermis, the dermoepidermal junction is greatly folded. Muscle fibres are also clearly seen to be present between the hair follicles.

### Postnatal d 10

Observation of the eye 10 d after birth reveals that the eyelids are still closed, with the line of fusion between them overlain with numerous hairs. An indentation is also now evident on the conjunctival side of the junction which corresponds to the depression seen on the external surface (Fig. 18). Keratinisation is also seen to be extending in from the epidermal surface into the region between the 2 eyelids and is also observed in the region of the indentation on the conjunctival surface. Numerous mature hair follicles separated by connective tissue and muscle fibres are the most characteristic feature of the dermis at this stage. Tarsal glands are also now clearly seen in the angle between the eyelid margins and the conjunctival surface.

### Postnatal d 12

By 12 d after birth, eyelid separation is all but complete, with squames of keratin now present throughout the junction (Figs 19, 20). At the margins of the 2 eyelids, the dermis of one eyelid is seen to be separated from that of the other by a basal layer of cells which is somewhat more regular in appearance on the conjunctival surface. Overlying the basal layers are 2–3 layers of clearer cells, the more superficial of which contain keratohyalin granules. Keratinisation is now more extensive on the conjunctival surface than before, but still only extends for a short distance along the surface. Thereafter, the conjunctivum consists of stratified squamous nonkeratinised epithelium. Tarsal glands are now well developed within the dermis; early evidence of duct formation is also
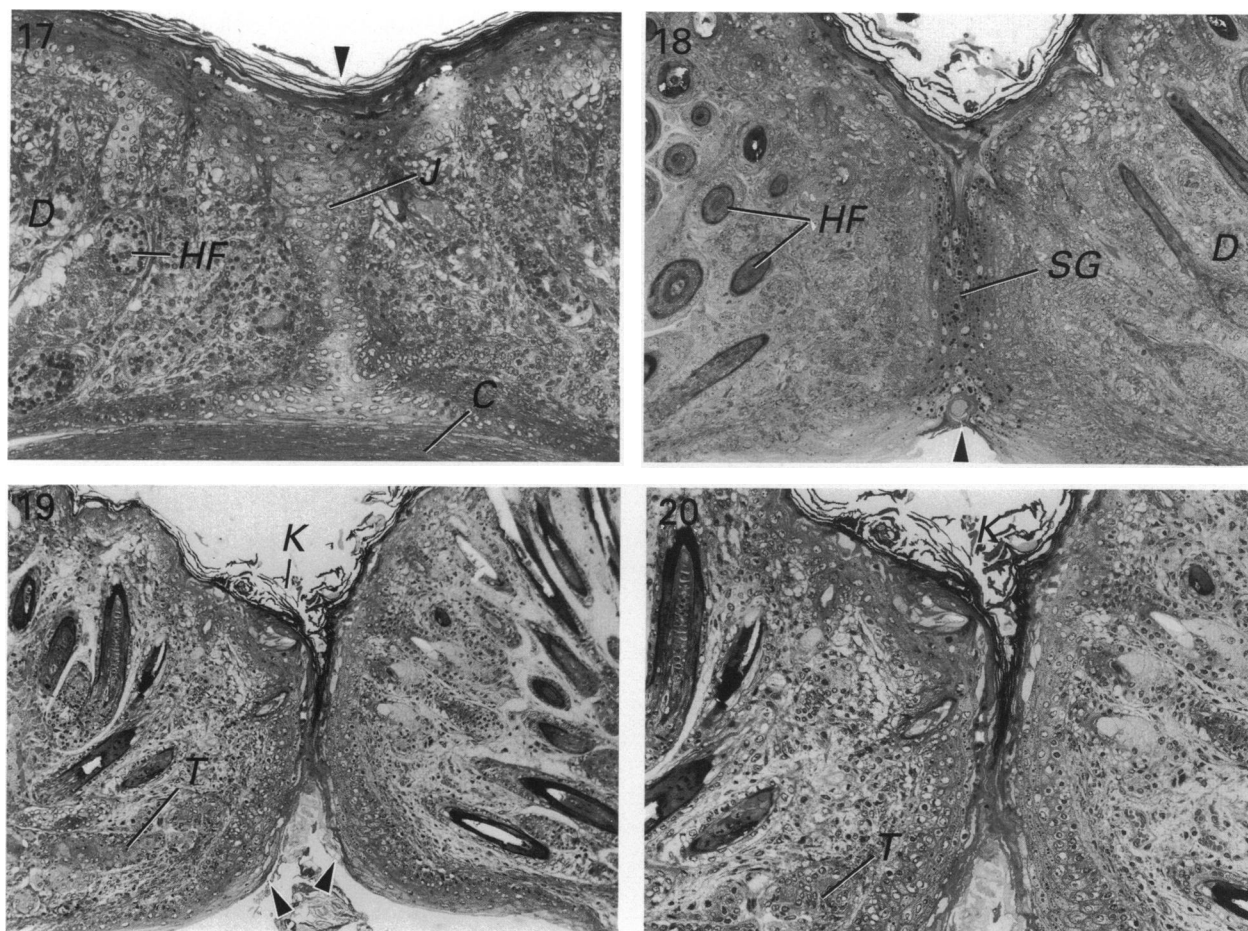
Fig 17. 5 d postnatal development. A shallow depression (arrowhead) is visible on the external surface overlying the junctional region (*J*). Hair follicles (*HF*) are visible in the dermis (*D*). *C*, cornea. × 160.

Fig. 18. 10 d postnatal development. A corresponding depression is now present (arrowhead) on the conjunctival surface of the fused eyelids. An extensive stratum granulosum (*SG*) is present in the junctional region. Mature hair follicles (*HF*) containing hair shafts are present in the dermis (*D*). × 160.

Figs 19 and 20. 12 d postnatal development. Separation of the eyelids is all but complete. Keratin layers (*K*) now extend downwards between the 2 eyelids. In Figure 20, the epidermis between the separating eyelids can be seen to consist of stratified squamous keratinised epithelium. Keratin (arrowheads) extends only for a short distance along the conjunctival surface. Thereafter the conjunctival surface consists of stratified squamous nonkeratinised epithelium. Tarsal glands (*T*) are visible adjacent to the free margin of each eyelid. Figure 19, × 100; Figure 20, × 160.

apparent. The latter open at the free margins of the developing eyelids.

## DISCUSSION

The processes involved in eyelid development are advanced by approximately 2 d in the mouse compared with the rat (Addison & How, 1921). The times at which these processes occurred in this study corresponded very closely to those reported by Harris & McLeod (1982). The observation that animals of the same gestational age were at different stages of development is also consistent with findings for both eyelid development in the rat (Addison & How, 1921) and limb development in the mouse (Maconnachie, 1979) and is a well recognised phenomenon in rodent

development (Theiler, 1989; Kaufman, 1992). However, a distinct progression could still be identified from early development through to eyelid closure and subsequent reopening, although the precise timing of these events could not be determined. Despite this, it was still possible to gain an appreciation of the rate at which different processes occurred.

The most striking feature of eyelid development and closure is the rapidity of events. At the earliest observed stages of development, seen at about 15.5 d p.c., the cornea of the eye is clearly visible with the primitive eyelids represented by protruding ridges of epithelium at its periphery. By the end of the next 24 h, eyelid formation has progressed to a point where the cornea of the eye can no longer be seen, being completely covered by the fused eyelids. The ap-

pearance of the eyelids during this time is of a rapidly proliferating epithelium with cells of each eyelid streaming across the corneal surface of the eye towards each other. Overlying the epithelium forming the eyelids is a layer of flattened cells termed the periderm by Bonneville (1968). Peridermal cells are characteristically found on the epithelial surface of the fetus, and are continuous with the lining of the amniotic cavity. At the edges of the gap, the peridermal cells are seen to be piling up on each other and produce a substantial cellular excrescence which overlies the line of apposition of the 2 eyelids.

The initial junction at the site of apposition of the 2 eyelids consists of a loose grouping of cells with obvious intercellular spaces present between them. As development continues, and the surface epithelium becomes stratified, the intercellular spaces disappear and the junctional zone becomes much more organised. In the human, desmosomes and gap junctions are found between the epidermal cells in the junctional zone (Anderson et al. 1967). This observation, if confirmed in the mouse, indicates that actual fusion does occur across the junction. On both the external surface and on the conjunctival surface, epithelium is seen to be continuous across the junctional zone. Keratohyalin granules are present and restricted to the cell layer immediately beneath the periderm but at this stage are never found on the conjunctival surface.

Once epithelial fusion has taken place, the dermis continues to develop, causing it to extend in towards the junctional zone. It is during this period when the eyelids are fused, which lasts from 15.5 d p.c. to approximately 5 d of postnatal development, that eyelid structures start to differentiate. Thus tarsal glands form during this period, although their openings into the free margins of the eyelids are not seen until eyelid separation is almost complete.

The development of hair follicles in the mouse follows the same pattern as that in the rat (Addison & How, 1921). The first follicles appear just after eyelid closure is completed, somewhat distant from the junctional zone. Further follicle development takes place towards the junction until, at the start of eyelid separation, hairs are present on the outer surface of the eyelid whereas follicles arising in the epithelium of the junction show only the earliest signs of hair shaft formation. Addison & How (1921) emphasised the role of developing hair follicles, especially in the junctional zone, in the eventual separation of the eyelids. In the mouse, although hair follicle development is well established in the junctional region, it appeared to play only an insignificant part in eyelid separation.

At as early as approximately 15.5 d p.c., and before eyelid fusion occurs, undifferentiated mesenchymal cells are seen in the area where muscle fibres subsequently develop. However, by about 16.5 d p.c., after eyelid closure has occurred, definitive muscle fibres are found, though only in the periphery of the eyelids, distant to the region of eyelid fusion. At no time, however, is muscle seen adjacent to the region of eyelid fusion.

The first sign of eyelid separation is the appearance of a slight depression on the external surface opposite the epidermal plug which extends between the 2 eyelids. This initial depression appears to result from a continuing enlargement of the dermis on each side of the apparently fixed epidermal plug in the junctional zone. Subsequent deepening of the groove results from a progressive keratinisation of the epidermal cells located between the 2 eyelids. Separation of the 2 eyelids by the desquamation of keratin squames progresses from the epidermal surface at the zone of apposition towards the conjunctival surface. A corresponding depression is first evident on the conjunctival side in this location at about 10 d after birth. Keratinisation then appears to extend onto both the conjunctival and epidermal sides until final separation occurs at around 12 d after birth.

The processes described here from eyelid development, through eyelid fusion to their subsequent reopening, are all observed in the human fetus, although the time course of the entire process is clearly much more protracted. Sevel (1988) divided eyelid development into 5 stages: (1) development of eyelid folds, (2) stage of eyelid fusion, (3) development of specialised features, (4) eyelid separation and (5) eyelid maturation. The same stages of eyelid development were observed in the mouse although the timing of events is measured in days as compared with weeks in the human. However, bearing in mind this time difference, there is a close similarity in the relative duration of each stage of development. The shortest stage in both the mouse and human was that of eyelid fold development. The longest stage was from the time of eyelid fusion through to the time when the eyelids finally separate. It is during this latter stage in both the mouse and human that the specialised structures of the eyelids are developed. Addison & How (1921) also analysed the development of the retina and compared the timing of retinal maturation with eyelid development. They suggested that eyelid fusion had a protective role protecting all components of the eye, but particularly the cornea, from exposure to potentially harmful substances in the amniotic fluid until the most critical stages of its differentiation are

completed. In the mouse, where the stage of development achieved at birth is only equivalent to that seen at midgestation in the human fetus, this is very approximately reflected in the timing of eyelid reopening. It would not be altogether surprising, therefore, if the degree of differentiation of the cornea and other intraocular structures reflected the poorer degree of differentiation of the mouse compared with the human conceptus at the time of birth.

It is also apparent that eyelid development corresponds closely to the development of epidermal structures elsewhere in the body. The timing of events in digit fusion in the mouse (Maconnachie, 1979) correlates very closely with those of eyelid fusion. Digit fusion and eyelid fusion both occur at approximately 15 d p.c. and start to separate 3–5 d after birth. Along the line of fusion a peridermal cell proliferation occurs which is lost at about 18 d p.c. when the periderm generally is sloughed off from the underlying epidermis.

The precise mechanism underlying digit and eyelid separation is still unclear. However, it has been shown that a polypeptide, epidermal growth factor (EGF), enhances both epidermal growth and maturation (Cohen, 1962). It has also been shown that EGF, when used in tissue culture medium, can enhance the lifetime and ability to multiply of human keratinocytes (Rheinwald & Green, 1977). Green (1977) found that the rate of squame production and shedding of epidermal keratinocytes in tissue culture was increased by 2–5 times in the presence of EGF. When administered within the first 3 d after birth, EGF, in the rat, was found to advance eyelid opening by up to 6 d (Birnbaum et al. 1976; Hoath, 1986). The effect of EGF was reduced the longer the time after birth it was given, causing Birnbaum et al. (1976) to conclude that rapidly growing epidermis is more sensitive to EGF than adult epidermis. It is therefore possible that the development and growth of the epidermis and its associated structures result from the rising levels of EGF in the embryo maximally affecting the rapidly proliferating epidermis.

Another factor which has been shown to affect the timing of eyelid opening is the degree of environmental stimulation to which a newborn rat is subjected. Smart et al. (1990) showed that the eyelids of rat pups reared in an 'enriched' environment opened ∼ 0.5 d earlier than those raised in an impoverished environment. To complicate further the issue of eyelid opening is the observation by Smart et al. (1986) that the left eye of rat pups reared artificially consistently opens earlier than the right eye. Why this should be is unclear. The authors suggested that it may be as a

result of the indirect stimulation that artificially reared animals receive.

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