MORPHOMETRIC STUDIES OF NORMAL AND ABNORMAL PRIMARY PALATE FORMATION IN NONCLEFT AND CLEFT LIP STRAINS OF MICE

BY

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ABSTRACT

Growth and development of the primary palate in the human embryo is complicated. Failure of the fusion of the maxillary prominence, with the lateral nasal and the medial nasal prominences results in cleft lip. Although many qualitative descriptions have been published on both normal and abnormal palatogenesis, there is little definitive information about mechanisms of primary palate formation. This study will address mechanisms using the mouse as the experimental model. The embryogenesis of primary palate of mouse is similar to that of human and spontaneous cleft lip is associated with genotype in both. Therefore the mouse provides an experimental model for studying primary palatogenesis.

The purpose of my research was to compare primary palate development in different strains of mice. Two of the strains studied, BALB/cByJ and C57BL/6J, have normal primary palate development, and three of the strains studied, A/J, A/WySn and CL/Fr, have stable frequencies of cleft lip. The first part of this study was to determine the cleft lip frequency and the resorption rate of the three cleft lip strains of mice. The results confirmed previous observations that A/J has a lower frequency of cleft lip and a higher resorption rate than that of A/WySn and CL/Fr.

The purpose of second part of this study was to determine the stage of body development and the chronological age at which primary palatogenesis takes place in these five strains. The results showed that in each strain, the number of tail somites was highly correlated with development of primary palate

in that strain. Somite development in the strains with genetic cleft lip liability was approximately twelve hours later than in the C57BL/6J strain. However, in the noncleft BALB/cByJ strain, the somite was also twelve hours later than in C57BL/6J. Thus, delayed chronological development appears not to be a factor contributing to cleft lip malformation.

In the third part of the study, the phases of primary palatogenesis were compared in the noncleft and cleft lip strains. The results showed fusion of the epithelia to form the nasal fin and replacement of the epithelial seam by mesenchyme was delayed in cleft lip strains relative to tail somite stages. The strains with higher cleft lip frequency, A/WySn and CL/Fr, were more delayed than the A/J strain with lower cleft lip frequency. The time of replacement of the nasal fin by mesenchyme occurred at about 12-13 tail somites in noncleft strains, 14 tail somites in A/J, 15 tail somites in A/WySn and 16 tail somites in CL/Fr. Forward growth of the maxillary prominence had the same pattern as mesenchymal replacement in the strains. The position of the maxillary prominence was highly correlated with the size of the nasal fin and the mesenchymal component in all strains. One major gene could explain this delayed formation in cleft lip strains and maternal effect could explain the more delayed formation in A/WySn and CL/Fr than in A/J strain.

The purpose of the fourth part of this study was to determine the tail somite stage when the oronasal membrane ruptures to form the primary choana dorsal to the primary palate. A definitive primary palate is established as a consequence of this rupture. Primary choana formation occurred at 18 tail

somites in CL/Fr and 20 tail somites in C57BL/6J. This earlier occurrence in CL/Fr than in C57BL/6J appears to provide a more limited developmental interval for mesenchymal replacement and enlargement in the cleft lip strain. A multifactorial threshold model was suggested from this study. The tail somite stage of mesenchymal replacement is applied as a scale of liability for the cleft lip malformation. Unfavorable growth may move embryos toward the threshold and result in an increased incidence of cleft lip. The threshold may be affected by the timing of primary choana formation.

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INTRODUCTION

The palate develops from two primordia: the primary palate and the secondary palate. Palatogenesis begins toward the end of the fifth week after conception in humans and is not complete until about the twelfth week. The secondary palate closes later in development than the primary palate. Genetic and environmental factors that influence its closure are different from those that influence the primary palate. Abnormal development of the primary palate, leading to a cleft lip, may interfere secondarily with secondary palate closure. Thus, on both embryologic and genetic grounds, congenital cleft lip (CL) and cleft lip with cleft palate CLP, appear to be etiologically related [in data combining the two they may be designed CL(P)]. Isolated clefting of the secondary palate (CP) is an etiologically independent entity (Fraser and Baxter 1954; Trasler and Fraser, 1963; Woolf *et al*, 1963; Fraser, 1970).

Cleft lip with or without cleft palate [CL(P)] is one of the most common craniofacial congenital malformations affecting primary palate development in human embryos. There are a large number of syndromes in which CL(P) may be one of the features. For most of these the cause is unidentified. A few are associated with recognizable chromosomal aberrations, and about a third are caused by major mutant genes. Each of these syndromes is rare, and together they may account for perhaps 5% of all cases. Most cases of CL(P) without associated malformations in humans appear to be multifactorially determined (Fraser, 1970).

There are striking differences in the CL(P) frequency between races: North American Indians in British Columbia have very high frequencies (2.75/1000) (Lowry and Renwick, 1969); Orientals have relatively high frequencies (1.7/1,000 births) (Kobayashi, 1958; Neel, 1958), Caucasians are intermediate (1/1,000) while Blacks tend to have low frequencies (0.4/1,000) (Chung and Myrianthopoulos, 1968; Khoury *et al*, 1983). Although the etiology of nonsyndromic CL(P) remains unknown (Fraser, 1989), these differences persist in different geographic regions, suggesting that they do not result from environmental alternations. It is thought that they may be associated with differences in face shape (Fraser and Pashayan, 1970). Because the multifactorial threshold model, to be discussed latter has been applied to cleft lip (Carter, 1969; Fraser, 1970, 1980), it would be useful to identify some biological attribute of liability such as face shape, that could be an indicator of increased risk.

1. Review of the literature

To provide a background for these studies, the results from previous investigations are presented comprehensively. This includes a review of normal development of the primary palate and the cleft lip malformation in human as well as animal models. The genetic causes of cleft lip in human and mouse are discussed. As three mouse strains used for these studies (A/J, A/WySn and CL/Fr) are genetically predisposed to CL(P) and susceptible to environmental effects, the effects of teratogens have also been addressed.

A. Normal development of the primary palate

I. The origin of facial mesenchyme

Before any primary palate formation occurs, neural crest cells migrate from the neural tube to the craniofacial region to form mesenchyme of the facial prominences. The importance of the role of the neural crest cells in forming the mesenchyme of the facial prominences was investigated by Johnston (1964, 1966) who conducted two significant experiments in chick embryos on this topic. In the first experiment small segments of neural crest from either mid- or forebrain were labelled with tritiated thymidine in donor embryos, and were then implanted in the corresponding region of host embryos. The results indicate that these cells made a significant contribution to the formation of the facial mesenchyme. In a second experiment, segments of the midbrain neural crest were removed prior to cell migration, and the embryos were then incubated for an additional period. This resulted in severe facial malformations which included an absence of a pronounced fronto-nasal prominence and a mandibular prominence. Removal of the forebrain neural crest, which normally makes a lesser contribution to facial mesenchyme, frequently resulted in the clefting of the primary palate only.

Using interspecific grafts of the neural primordium between quail and chick embryos, Le Lievre and Le Douarin (1975) have shown that the mesenchyme of the maxillary prominence and the branchial arches is composed of mesectodermal cells. Noden (1975, 1983) has demonstrated that the avian crest cells from the posterior mesencephalon migrate en masse away from the neural tube between the epidermis and the underlying mesoderm. Then this

neural crest population migrates and proliferates throughout both the region ventrolateral to the mesencephalon and the future maxillary prominence. Neural crest cells migrating from the anterior mesencephalon and posterior diencephalon move rostrally, and at later stages they overlap with those derived from the posterior mesencephalon, contributing to the formation of the maxillary prominence. Nichols (1986) has shown that in mouse embryos, neural crest formation and emigration at midbrain-rostral hindbrain neural folds are complete at late 4 to 5 somites of development. The neural crest leaves behind overlying squamous epithelium. At approximately 10 somites of development, this neural crest mesenchyme is distributed dorsolateral to the pharynx and displaced ventromedially in a narrow, transient subectodermal space functionally similar to that observed in the chick embryo. If the fate of the crest mesenchyme in the mouse is similar to those in birds and amphibians, this mesenchyme will form bone, cartilage and connective tissue of the first branchial arch.

II. Induction of the nasal organ

The induction of the nasal organ starts primary palate formation. At about 33 days (stage 15) after conception in humans, the area which will form the nose becomes induced and begins to elevate forming the medial and lateral nasal prominences. In the Salamander, nasal organ induction involves a succession of different inductors. In the gastrula and the neurula stages portions of the endoderm and mesoderm act as inductors. The final inductor of the nasal organ is a portion of the central nervous system. (Jacobson, 1963a, 1963b).

The mesenchyme underlying the ectoderm of the facial region originates from the neural crest cell population (Johnston, 1964, 1966; Le Lievre and Le Douarin, 1975). Its high rate of proliferation (Minkoff and Kuntz, 1977, 1978) is thought to be maintained by an epithelial-mesenchymal interaction. Movement of mesenchymal tissue from the medial nasal prominence into the base of the nasal groove and into the medial area of the lateral nasal prominence was observed when ^{3}H thymidine was implanted with a sable hair probe (Patterson *et al*, 1984; Patterson and Minkoff, 1985). A series of separation and recombination experiments involving a variety of tissue configurations in organ cultures from chick (Saber *et al*, 1989) suggested that the influence of the epithelium on mesenchyme viability was stage dependent, and epithelial-mesenchymal interactions appear to evoke, within the mesenchyme, a zonal growth-sustaining effect. In an effort to determine whether epithelial-mesenchymal interaction may also be relevant to the maintenance of the growth rates in the facial prominences, Bailey *et al* (1988) have found that cells located deeper within the mesenchyme have lower proliferation rates than those closer to the epithelium in the chick. During the latter stages of development, however, this trend is not observed. The reason for this mechanism is still unknown.

There are many theories as to the kind of macromolecules involved in epithelial-mesenchymal interaction. The presence of serotonin uptake sites in the epithelia and the serotonin binding protein in the underlying mesenchyme raises the possibility that serotonin might be involved in epithelial-mesenchymal interaction (Lauder *et al*, 1988). Xu *et al* (1990) have analyzed the distribution of

a group of macromolecules associated with the basement membrane in the developing primary palate in chick embryos. The results indicate that the regional differences within the maxillary prominence and between the maxillary prominence and adjacent regions, such as the roof of the stomodeum, are related to developmentally regulated changes. These changes are associated with the presence and distribution of type IV collagen. Type IV collagen expression was decreased in actively growing regions, i.e. regions of maxillary outgrowth on the lateral surface.

III. Epithelial fusion

At about 37 days after conception in humans (Stage 16), after the facial prominences become induced to form the nasal placode, the lateral wall of the nasal placode is formed caudo-occipitally by the maxillary prominence and cranio-frontally by the lateral nasal prominence. Its medial boundary consists of the medial nasal prominence, which contacts the maxillary prominence in the frontal portion of the nasal groove and the lateral nasal prominence in the caudal portion. The area of contact is called the epithelial plate or nasal fin (Streeter, 1948; Vermeij-Keers, 1972) and it forms the continuity between the nasal cavity and the roof of the mouth. This continuity between the nasal sac and the roof of the mouth becomes interrupted and replaced by the active proliferation of the mesenchyme of maxillary prominence and nasal prominences at stage 17 (Streeter, 1948; Warbrick, 1960; Vermeij-Keers, 1972; Diewert and Shiota, 1990; Diewert and Van der Meer, 1991).

In the mouse embryo, the development of the primary palate starts at about 10 days and 18 hours (Reed, 1933; Trasler, 1968). From the time of their appearance the lateral nasal and medial nasal prominence are connected by an isthmus (Trasler, 1968). Fusion of lateral nasal and medial nasal prominences commences from the back portion of the mouth and proceeds ventrally. Trasler (1968) showed that at the "crescent" face stage, the epithelium of the medial and lateral nasal prominences begins to make contact in a posterior to anterior direction forming a flat plate of double epithelium called the nasal fin. A "zipping up" process follows this fusion of epithelia giving the nasal opening a comma shape.

The initial contact between the cells of the medial and lateral nasal swellings is made by short projections from one superficial cell to the surface of an opposing superficial cell in the mouse embryos (Gaare and Langman, 1977a). Trasler and Ohannessian (1983) have also shown that cells approaching or in contact with opposing cells form cell projections, intercellular junctions, desmosomes, and microfilaments, demonstrating firm contact between the opposing epithelia. Millicovsky and Johnston (1981) have shown that in the mouse embryo, epithelial cells lose their surface microvilli before contact. After a brief period of quiescence, they begin to fill the groove separating the facial prominences by producing a series of surface projections that increase in size and complexity as the process of fusion progresses.

The carbohydrate surface coat has been suggested to be an essential factor in mediating adhesion between opposing palatal shelves (Greene and Pratt, 1976). When these investigators inhibited surface coat production by means of diazo-oxo-norleucine (DON) in vitro, the palatal shelves failed to fuse. A cell surface coat is also found over the epithelial linings of the nasal prominences in the region of presumptive fusion using ruthenium red and radioactive precursors (Gaare and Langman, 1977a; Figueroa and Pratt, 1979). Burk *et al* (1979) found the concentration of surface coat material on the epithelium of the presumed fusion area to be higher than other regions of the nasal folds using ^{3}H-Concanavalin A. These findings support the hypothesis that the cell surface coat is associated with the ability of epithelial shelves or folds to adhere and fuse. In fact, complex carbohydrates have for some time been implicated in cell aggregation and intercellular adhesion in various in vitro cell systems (Pessac and Defendi, 1972; Oppenheimer, 1973; Roseman, 1974; Greig and Jones, 1977).

IV. Mesenchymal replacement

At about 41 days after conception in humans (stage 17), a major portion of the nasal fin is interrupted by the active proliferation of the mesenchyme from the lateral nasal, medial nasal and maxillary prominences. This mesenchyme connects the medial and lateral walls of the nasal groove and establishes the primordium of the palate (Streeter,1948; Warbrick, 1960). There are two concepts about mechanisms of replacement of the nasal fin by the mesenchyme. One of

these is fusion between the facial prominences which is similar to that in formation of the secondary palate. As the two palatal processes come together, the covering epithelial layers are brought into contact. An epithelial seam forms, and shortly thereafter the epithelial seam begins to fragment as the cells either degenerate (Greene and Pratt, 1976) or transform into mesenchyme (Fitchett and Hay, 1989). In contrast with fusion is a series of events which was named merging by Patten (1961). In merging, mesenchymal growth and migration underlying the epithelium of the prominences eliminates the intervening epithelium. The epithelium is pushed out from between the elevations instead of being apposed and then disintegrating or transforming as in fusion.

Töndury (1950) noticed in the anterior part of the epithelial plate degenerative processes expressed by the occurrence of pyknotic nuclei and nuclear fragments followed by destruction of the basement membrane. In contrast, Anderson and Matthiessen (1967) interpreted these nuclear fragments as peripherally sectioned mitotic figures. In addition, they did not detect histiocytes, which they postulate to be present wherever embryonic epithelium disappears. Therefore, they were inclined to agree with Patten (1961) who explains the disappearance of the epithelial plate by a process called merging, in which the epithelium between two swellings is squeezed out by pressure exerted by the underlying mesenchyme upon the epithelium of the groove. Vermeij-Keers (1972) found that the basement membrane of the epithelial plate had disintegrated locally. Between the normal epithelial cells in this plate nuclear fragments were found. The limited number of human embryos of the relevant

stages available for these studies limited definite conclusions.

Recently, Diewert and Van der Meer (1991) quantified growth of the epithelial nasal fin and mesenchymal replacement of the nasal fin during normal human primary palate formation. Thirty serially-sectioned human embryos of stage 16 to 19 in the Carnegie Collection (Streeter, 1948; O'Rahilly and Müller, 1987) were studied. The results showed that during stage 16 the nasal fin formed between the medial nasal and maxillary prominences. During stage 17, a mesenchymal bridge formed through the nasal fin, and the size of the mesenchymal bridge increased rapidly to occupy up to 50% the total area. During stages 18 and 19 total primary palate area increased and the mesenchymal bridge enlarged to constitute 65 to 85% of the total area.

Gaare and Langman (1977b) reported that in mouse embryos, shortly before the epithelial linings of the opposing nasal prominences make contact, cell degeneration characterized by condensation and fragmentation occurs in the epithelial linings of the prospective fusion areas. After fusion has established the nasal fin, epithelial cells continue to degenerate in the same manner. However, cell degeneration can not account for complete regression of the nasal fin, since many morphologically healthy epithelial cells are always present. They suggested that these surviving epithelial cells incorporate into the adjacent epithelial linings of the expanding primary nasal and oral cavities.

The interchange of tissue phenotype, especially epithelial to mesenchymal, is a common phenomenon during early embryogenesis. It is possible that there exists in most epithelia, a readily triggered mechanism that

turns on the mesenchymal genetic program. Greenburg and Hay (1982, 1986, 1988) demonstrated that a variety of adult and embryonic epithelia that normally do not give rise to mesenchyme do so when the isolated tissue is immersed inside hydrated Type I collagen gels. Confronted with collagen fibrils in close contact on all sides, these well-established epithelia express the potential for tissue-type conversion in response to an abnormal extracellular matrix environment.

Fitchett and Hay (1989) showed that palatal medial edge epithelium is an ectoderm that retains the ability to transform into mesenchymal cells. They report that cell death is not the major mechanism leading to removal of the midline epithelial seam created by contact of the two palatal shelves. Rather, opposing basal cells adhere, after sloughing of the periderm, proliferate, and then transform into mesenchyme. The basal lamina disappears as basal cells extend filopodia and then pseudopodia into the adjacent connective tissue compartment. The glycogen rich basal cells have euchromatic, vesicular nuclei and abundant rough endoplasmic reticulum. Before they begin to elongate and move into the extracellular matrix, they acquire a vimentin-rich cytoskeleton and lose keratin expression. Vimentin filaments are the characteristic intermediate filament type of mesenchymal cells. The changes in cell shape and cytoskeleton are similar to those reported in already established epithelial mesenchymal transformations (Hay, 1968: Bernanke and Markwald, 1979, 1982; Nichols, 1981; Franke *et al*, 1982).

V. Primary choana formation

At about 44 days after conception in humans (stage 18), the width of nasal septum and medial nasal prominence decreased to 0.5 to 0.7 times that of stage 16 (Diewert *et al*, 1989; Diewert and Lozanoff, 1989, 1990). At the same stage, the nasal fin dorsal to the zone of mesenchymal penetration persists and cavitates, forming the oronasal membrane which separates the nasal pit from the cavity of stomodeum. The rupture of the oronasal membrane is brought about by the disintegration of the cells that form it. This results in the opening of a respiratory passage from the nostril through the primary choana to the pharynx (Streeter, 1948; Warbrick, 1960).

In the mouse embryo, after the disintegration of nasal fin and penetration of mesenchyme, a portion of the nasal fin remains at the back of the nasal pit forming the oronasal membrane, with the formation of interstitial gaps occurring at the 11th day after conception (Trasler, 1968; Tamarin, 1982). The gaps enlarge and coalesce so that a completely patent opening between nasal passage and stomodeum is established by 13 days. The membrane consists of two layers of simple squamous epithelium which become separated as involution progresses. The form of the choanal antrum changes from a simple funnel-shaped ellipse early in the 13th day to a complex slit like opening within the following 24 hours. This coincides with the completion of a definitive primary palate and the enlargement and elevation of secondary palatal shelves.

B. Abnormal development of the primary palate and cleft lip malformation

I. Morphogenesis of cleft lip in the human embryo

Cleft lip is a result of failure of fusion between the medial nasal prominence, lateral nasal prominence and/or maxillary prominence. One possible cause of this defect is that the ventral ends of the prominences fail to come into actual contact with each other for fusion. This can occur if growth is defective in either or both prominences. Töndury (1964) described an embryo with unilateral complete cleft lip where the primary cause for the faulty development appears to be defective growth of the lateral nasal prominences. Another explanation is that the nasal fin persists throughout the developmental stages preventing the mesenchyme of the maxillary and fronto-nasal prominence from making contact. Subsequently, when the dorsal part of the nasal fin undergoes the normal cavitation and cleavage, resulting in the formation of the primary choana, the ventral part of the nasal fin also undergoes cleavage resulting in the formation of cleft lip (Stark, 1954; Warbrick, 1960).

Anderson and Matthiessen (1967) have suggested that complete cleft lip will also appear if mesenchymal proliferation is retarded in the medial nasal and maxillary prominences, and incomplete cleft lip will appear in cases with a less marked retardation of the mesenchymal proliferation in the above mentioned mesenchymal centers. Furthermore by investigating human embryos with primary palatal clefting in the Kyoto collection, Diewert and Shiota (1990) showed deficient mesenchymal bridge growth and a visible deficiency of tissue in the cleft areas. The results also showed regional growth deficiency or developmental

abnormality in the palatal tissues during the critical time of rapid mesenchymal bridge enlargement in cases of partial or incomplete clefting.

II. Morphometric study of postnatal human cleft lip

Fraser and Pashayan (1970) have shown that parents of children with cleft lip tend to differ from the general population in certain dimensions of facial topography. There was a significant tendency for the anterior surface of the maxilla to be flatter in the experimental group than in the controls. In addition, in the experimental group the mean dizygomatic and intraocular chin measurements were larger, the frequency of rectangular and trapezoid shapes was higher, and the upper lip was less protuberant relative to the lower lip. Coccaro *et al* (1972) have also shown that parents who lack facial deformities, but have cleft lip and palate children, have faces that are less convex with a tendency toward mandibular prognathism. Vertical and horizontal measurements of the upper face and the nose length were found to be shorter for parents of cleft lip and palate children.

Erickson (1974) analyzed three proposed microforms in the normal sibs of children with cleft lip but with or without cleft palate: (1) facial profile, (2) dental arch shape, and (3) palatal form. It is concluded that the sibs of these facially malformed children are likely to be different from normal children. However, these differences are not sufficient to classify these people as a group having a distinct malformation with any degree of certainty.

Studies of facial morphology in monozygotic twins discordant for CL(P) suggest that approximately two thirds of cleft lip cases are caused by underdevelopment of the medial nasal prominences (Johnston and Hunter, 1989). The twin studies indicated that the remaining one-third of cleft lip cases result from underdevelopment of the maxillary prominences. Thus, Johnston and Hunter (1989) proposed the existence of two major CL(P) groups; one with small medial nasal prominences in the other with small maxillary prominences.

III. Morphogenesis of cleft lip in laboratory animals

Reed (1933) has proposed that harelip in animals is due primarily to the lateral nasal prominence and the medial nasal prominence failing to fuse. This failure is probably due to a retarded growth rate of the maxillary prominence. Trasler (1968) has shown that formation of the normal lip requires the posterior portions of the medial and lateral nasal prominences to remain continuous with each other and with the medial portion of the maxillary prominence. In an A/J embryo genetically predisposed to clefting, the medial nasal prominences do not diverge laterally as much as they do in an embryo that is not predisposed. This results in a decrease or failure of epithelial fusion between the medial and lateral nasal prominences, and consequently, a lack of consolidation of the isthmus then occurs.

The hypothesis that face shape is a causal factor in genetic predisposition to cleft lip in mice was further tested by Juriloff and Trasler (1976). Their results from measuring photographs of embryos support this hypothesis.

However, it is suggested that the susceptibility to another type of cleft lip in other genotypes could arise for example, through hypoplasia of one of the facial processes. Trasler and Machado (1979) have found that a particular facial complex is associated with cleft lip predisposition. Premaxillary length is significantly shorter in newborns and adults in mouse cleft lip lines CL/Fr, A/J and L than non-cleft lines M and C57BL/6J. Premaxilla width also tended to be narrower in the adults, and gum length, in newborns and adults, tended to be shorter in cleft lip lines.

Developmental alterations associated with spontaneous cleft lip and palate in CL/Fr mice (Millicovsky *et al*, 1982; Forbes *et al*, 1989) include: altered facial geometry, in which the orientation of the medial nasal prominences is almost parallel to the mid-sagittal plane, depressed ability of the surface epithelium of primary palate primordia to participate in the fusion process, and hypoplasia of the lateral nasal prominences. Ohbayashi and Eto (1986) have suggested that the medial nasal prominences (MNP) play a critical role in normal facial development and cleft lip formation based on culture experiments done in vitro using rat embryos that have had a part of each facial prominence removed. They found that cleft lip like malformation was observed only in the MNP-excised group.

C. The genetic causes of cleft lip

I. One major gene in mouse cleft lip

The genetic causes of cleft lip have been controversial. In the 1930's,

Reed (1936) outcrossed CL(P)-liable stock to test if one single major essential gene for harelip was present. The results were consistent with the single major locus hypothesis. Reed noted, however, that it was also probable that cleft lip resulted from the cumulative effect of a small number of recessive genes containing the same genetic information. By backcrossing A/J and C57BL/6J mice, Juriloff (1980) showed that one or two loci were involved in the expression of CL(P). In a further study, Juriloff (1986) has produced a congenic strain, in which the cleft lip gene is transfered into an unrelated AEJ/GnRK strain. The stable frequency of cleft lip from detected carriers in each backcross generation is most compatible with the one locus mutation model. Biddle and Fraser (1986) have also proposed that the difference between the A/J mouse embryo and the C57BL/6J strain appears to be determined by a single recessive gene.

II. Maternal effects

Davidson *et al* (1969) demonstrated a maternal effect on the frequency of spontaneous cleft lip in the mouse. In repeated backcross studies of A/J and C57BL/6J the frequency of CL(P) was higher for genetically equivalent embryos from A/J mothers compared with those from hybrid mothers. The authors have suggested that clefting could be mediated through a genetically determined maternal uterine biochemistry or physiology. If this is so, then the genetic difference between the strains must be multifactorial. Alternatively, it is possible that the clefting strain mother did not provide a cytoplasmic factor, found in C57BL/6J, which provides resistance to clefting. Bornstein *et al* (1970) tested

these hypotheses and have shown that the maternal effect on cleft lip susceptibility was present in the CL/Fr strain, but it was not transfered through any cytoplasmic factor.

Juriloff and Fraser (1980) have found that the difference in CL(P) frequency between the A/J and CL/Fr strains was not determined by the embryo genotype but by the maternal genotype. Also the data shows a reciprocal relationship between cleft lip frequency and resorption frequency. This suggests that the maternal trait may cause a difference in the survival of cleft lip fetuses. Juriloff (1982) has repeated her study with A/WySn and A/HeJ and found the same result.

Maternal effects have been sought for human CL(P) data. Maternal genetic effects did not account for the racial differences in frequency of CL(P) in Hawaii (Ching and Chung, 1974). No evidence of maternal effects was found when comparing CL(P) recurrence risk between maternal half and paternal half siblings (Bingle and Niswander, 1977). Juriloff (1980) has suggested that if the mouse populations were as heterogeneous as the human populations, the maternal effects probably would not be revealed. The maternal effects were detected when the genetic risk of the embryos was sizable and constant. A more recent paper compared clefting frequencies in Blacks and Whites and reciprocal crosses (Khoury *et al*, 1983). The results showed that the difference in the reported rates of CL(P) between reciprocal crosses of Whites and Blacks is due to the effect of mother's race. When the mother is Black, offspring of White fathers did not have a higher rate CL(P) than those of Black fathers. This study

documents the existence of maternal determinants of CL(P) in humans.

III. Reciprocal relationship between cleft lip and resorption rate

In the study of genetic maternal effects on cleft lip frequency in A/J and CL/Fr mice, Juriloff and Fraser (1980) have shown a reciprocal relationship between cleft lip and resorption frequency of the two strains. In another study (Juriloff, 1982) this reciprocal relationship was observed to be directly related to the segregation of genetic variation. The existence of similar genetic variation in humans would partially explain inverse association between clefting and spontaneous abortion (Stein *et al*, 1975; Bear, 1978). The concept of "terathanasia" the natural abortion of defective embryos (Warkany, 1978) is also supported.

It has been thought that thyroxin may reduce the frequency of cleft lip (Woollam and Millen, 1960). However, by the administration of thyroxin, it has been shown that the reduced frequency of cleft-lip embryos collected at term after thyroxin treatment is due to their increased mortality rate, and not to prevention of the lip defect (Brown *et al*, 1974; Juriloff, 1981). A further study by Juriloff and Harris (1985) on the thyroxin-induced differential mortality of cleft lip in mouse embryos has also shown that following the thyroxin treatment cleft lip and normal embryos died, but cleft lip embryos died at higher rate. Therefore thyroxin does not affect the events that lead to cleft lip but the presence of cleft lip increases the liability for thyroxin-induced death.

IV. Uterine site effects

Trasler (1960) has found that within A/J strain, embryos in the uterine site nearest the ovary develop cleft lip significantly more often than embryos in other positions in the uterine horn. Kalter (1975) showed that the frequency of CL(P) was higher at the ovarian and cervical sites than elsewhere. The resorption was lower at the ovarian site than elsewhere, and this mortality trend ran along with fetal weight, ie, as the former increased the latter decreased. Juriloff (1980) has reinvestigated this uterine site effect and shown increased cleft lip and decreased resorption at the ovarian site. It is suggested that a relatively privileged area at ovarian site in both A/J and CL/Fr allows the survival of cleft lip embryos that would have died elsewhere (Juriloff, 1980).

V. General concept of the multifactorial threshold model

There are many characteristics of biological interest which appear to vary in a discontinuous manner but are not found to be inherited in a simple Mendelian fashion. These can be classified into two phenotypic classes, affected or not-affected. Characteristics of this sort appear at first sight to be outside the realm of quantitative genetics; yet when they are subjected to genetic analysis they are found to be inherited in the same way as the continuously varying characteristics.

"The clue to understanding the inheritance of such characteristics lies in the idea that the characteristics have an underlying continuity with a threshold which imposes a discontinuity on the visible expression, as depicted in Figure 1.

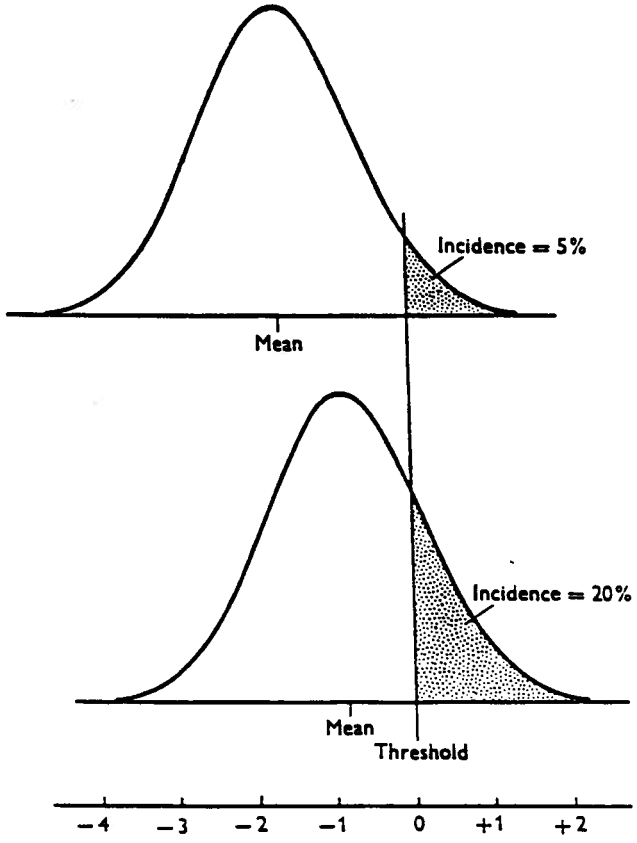


Fig. 1. Falconer's concept of threshold. Two populations or groups with different mean liabilities. The liability is normally distributed, with the same variance in the two groups. The groups are compared by references to a fixed threshold. The stippled portions are the affected individuals with the incidences shown (from Falconer, 1965).

When the underlying variable is below this threshold level the individual has one form of phenotypic expression, which is conceived 'normal'; when it is above the threshold the individual has the other phenotypic expression, i.e. 'affected'." (Falconer, 1965). For such characteristics with a threshold, the underlying continuous variable has been called the liability in the context of human diseases. The continuous variation of liability is both genetic and environmental in origin. It might be thought of as the developmental rate of a specific process, and thus, in principle, it could be measured and studied as a metric character in the ordinary way.

The classical threshold analysis is described in a paper written by Wright (1934) on polydactyly in guinea pigs. Three closely inbred guinea pigs (strains 2, 13, 32) with normal 3-toed hind feet were crossbred with strain D with polydactyly. The crosses between 2 and D simulate one factor Mendelian heredity to a remarkable extent in the dominance of 3-toe in F1, and apparent segregation in F2 in a fairly close approach to a 3:1 ratio and in the backcross to strain D in a 1:1 ratio. This interpretation breaks down in the tests of the supposed segregants. These tests indicate that there are at least 3 factors of comparable importance and more probably 4 by which strains 2 and D differ. There is a close approach to blending inheritance in a character which approaches alternative expression because of physiological thresholds. The crosses between 32 and D gave closely similar results to those of 2 and D. On the other hand, the crosses between 13 and D gave a very different result which indicates that strain 13 is much closer to the threshold for polydactyly.

A study was made by Grüneberg (1951) on the CBA mouse strain. These animals have abnormally small third molars, and some lack these teeth completely. It appears that the absence of the third molar occurs when the dental lamina, which forms the tooth bud, falls below a certain size. The difference between the mean third molar size of the CBA strain and that of C57BL is brought about by multiple genes. This result shows that if the F1 males were backcrossed to CBA females, there was a shift in the variable distribution of the offspring toward that of the CBA. In a later paper, Grüneberg (1952) has described quasi-continuous characters in the sense that the underlying genetic basis is a continuous variable, with multiple factor inheritance, which is divided by a physiological threshold into normal and abnormal animals. The peculiar genetical properties of quasi-continuous characters are regarded to be due partly to the fact that a continuous distribution may shift in relation to a physiological threshold, and partly that they share with ordinary continuous variables, the multiple gene basis and the sensitivity to influence of the environment. Green's study (1971) of presacral vertebrae and Tom *et al*'s study (1991) of exencephaly are further examples of this type of approach.

VI. Multifactorial threshold model and one major gene in human cleft lip

A multifactorial threshold model which accounts for the liability of the common, familial, human disorders was developed by statistical geneticists (Falconer, 1965). Using Falconer's procedure on cleft lip and palate, an additive polygenic model has been fitted to the cleft lip data from human populations

(Carter, 1969, 1976; Bixler *et al*, 1971, Woolf, 1971, Czeizel and Tusnady, 1972, Chung *et al*, 1974; Bear, 1976).

From the analysis of Danish and Japanese families of probands with CL(P) Chung *et al* (1986) found that the Danish data is best explained by the mixed model, which combines the major gene and multifactorial inheritance models. On the other hand, the Japanese data is best accounted for only by the multifactorial inheritance model. These findings appear to explain the puzzling observation that the Japanese population which has a higher general incidence of CL(P) has a lower recurrence risk of having CL(P) relative to the Caucasian population (Chung *et al*, 1986). In other words, factors outside the major cleft inducing gene have a greater effect on the Japanese population. The factors which affect one generation may not be present in the next generation, thus the risk for clefting in the second generation does not need to be the same as their parents. To identify the major gene, Ardinger *et al* (1989) have shown that either the transforming growth factor-α (TGF-α) gene itself, or other DNA sequences in an adjacent region, contribute to the development of a proportion of the cases of cleft lip in humans. Further evidence for an association between genetic variation in transforming growth factor α and cleft lip and palate has been shown by Chenevix-Trench *et al* (1991). However, in another study of seven families with CL(P) segregating in a dominant manner, the association of the A2B2C2 haplotype reported by Ardinger *et al* (1989) was not found, with none of the affected parents having this TGF-α haplotype (Hecht *et al*, 1990).

Studies of facial morphology in monozygotic twins discordant for CL(P) suggest that approximately two thirds of the cleft lip cases are caused by underdevelopment of the medial nasal prominences and the remaining one-third of cleft lip cases result from underdevelopment of the maxillary prominences (Johnston and Hunter, 1989). These groups have also been considered comparable to Chung *et al*'s (1986) multifactorial and single major gene groups, respectively. Marazita *et al* (1986) have studied cleft lip with or without cleft palate in the families of non-syndromic CL(P) probands who were surgically corrected. The data, which come from three populations, provide no support for the multifactorial threshold model but did provide evidence of the presence of a major gene responsible for cleft lip in at least a portion of cases.

Melnick *et al* (1980) have tested the multifactorial threshold inheritance model by studying 1,895 persons born in Denmark between 1941 and 1968. The individuals were born with cleft lip with or without cleft palate. The results revealed that neither the multifactorial threshold model nor the single-major locus model provided an adequate fit. As an alternative model, the monogenic-dependent susceptibility (MDS) to a variety of teratogens was proposed in light of experimental mouse and human data. In humans, a study by Bonner *et al* (1978) suggests an association between particular HLA haplotypes and clefting. However, Van Dyke *et al* (1980) have shown that it is very unlikely that spontaneous cleft lip with or without cleft palate is closely linked to HLA. In mouse, it has been shown that the maternal H-2 haplotype significantly affects the incidence of corticosteroid-induced isolated cleft palate (Bonner and Slavkin,

1975) and cleft lip with or without cleft palate (Silberman *et al*, unpublished, cited from Melnick *et al*, 1980). Juriloff (1982) showed that the H-2 gene region did not appear to influence CL(P) frequency on the A/WySn strain background.

D. Environmental effects on cleft lip malformation

I. Hadacidin

Hadacidin is an antibiotic isolated from broth culture of *Penicillium frequentants* (Chaube and Murphy 1963). It is a potent inhibitor of the enzyme, Adenylsuccinic synthetase in normal rat tissue in vitro, by competing with L-aspartate for the active site on the enzyme molecule (Shigeura and Gordon, 1962a, 1962b).

Lejour-Jeanty (1966) has shown that Hadacidin induces harelips in the rat which are comparable with human abnormalities. The effect of the drug is confined to the lateral nasal prominence, which seems to be responsible for the absence or incompleteness of fusion of both edges of the olfactory pit. The maxillary prominence does not take part in the fusion, but its slow and disorientated growth is thought to contribute to the maintenance of the nasal fin which persists or may be partially destroyed by the mesenchyme. Another study (Lejour, 1969) of cleft lip induced by Hadacidin in rats indicated that complete clefting is more often the result of a disruption of the contacting edges of the nasal groove rather than of an absence of fusion, as usual. Clefts with bridges are produced when the nasal fin is only partly penetrated by mesenchyme in the region of the maxillary arch.

II. 6-aminonicotinamide

Specific vitamin antimetabolites utilized in experimental mammalian teratology have been incoporated into deficient diets (Nelson, 1957) or injected into pregnant animals. (Wilson, 1959, 1964). The niacin antimetabolite, 6-aminonicotinamide (6-AN) has been shown to be teratogenic in various species (Murphy *et al*, 1957; Pinsky and Fraser, 1959). 6-AN has been shown to be an antimetabolite for nicotinamide (Pinsky and Fraser, 1960). It has been demonstrated (Dietrich *et al*, 1958) that 6-AN inhibits the diphosphopyridine nucleotide (D.P.N.) - dependent reaction by substituting the nicotinamide in the D.P.N. molecule and rendering the inactive analogue incapable of functioning in the hydrogen and electron transfer reactions essential to the normal metabolism of the cell.

Pinsky and Fraser (1960) found that cleft lip and cleft palate would result from an injection of 6-AN on day 9 1/2 followed by nicotinamide two hours later. However, no malformations were observed if treatment was given on day 10 1/2. They concluded that the study of the protective effect of vitamins against the teratogenic activity of their antagonists appears to be useful for analyzing the vitamin requirements of various organogenetic processes in the developing embryo. This shows that nicotinamide requirements of the embryo-mother system appears to vary from one-gestational period to another.

Trasler and Leong (1982) have found that treatment with 6-AN on day 9 produced 18% median cleft lip and no lateral cleft lip, whereas treatment on day 10 produced 22% lateral cleft lip and no median cleft lip in near-term C57BL/6

fetuses. A median cleft lip is associated with a critical reduction in growth of the medial nasal areas. Histologically, the nasal ectoderm of the treated group had fewer cells per unit area in the medial nasal area than of the controls, adjacent mesenchyme had an increase in the number of dense bodies, a possible result of cell death occurring, and a significant reduction of mitotic index in the nasal area. On the other hand, mechanisms for 6-AN-induced lateral cleft lip may involve failure of less organized denser nasal ectoderm to fuse, and a growth reduction of both lateral and medial nasal prominences. Histologically, the mesenchyme in this area also contained a large number of dense bodies, and the mitotic index was significantly reduced in both nasal and neural areas suggesting that mitotic inhibition may have caused the observed abnormalities.

In a further investigation, Trasler and Ohannessian (1983) made comparisons of the ultrastructure of cleft lip liable and control embryos treated with 6-AN. A few 6-AN-treated embryos showed abnormal contact that appeared malpositioned and tenuous. The teratogen also produced increased cell death and a denser epithelium and mesenchyme. The denser appearance of the mesenchyme may be associated with a decrease in the intercellular matrix as was found in another study by Flint and Ede (1978).

III. Phenytoin

The anticonvulsant phenytoin (PHT) is teratogenic to inbred strains of mice (Massey, 1966). Treatment of pregnant mice with PHT increases the frequency of cleft lip and/or palate in surviving fetuses (Johnston *et al*, 1978). The

first association of phenytoin with birth defects in humans was reported in 1968 (Meadow). A group of abnormalities in growth and performance have been characterized as the Fetal Hydantoin Syndrome (Hanson and Smith, 1975) and they include: craniofacial abnormalities, cardiac and limb defects, general deficiencies in growth, and mental retardation.

Martz *et al* (1977) studied the possibility that the teratogenesis of PHT is due to the arene oxide produced by the molecule. Arene oxide can form covalent bonds to gestational embryonic tissue and cause abnormal development. Scanning and transmission EM analyses of high incidence of cleft lip and palate produced by maternal intraperitoneal administration of phenytoin on gestational day 10 in A/J mice have been reported by Sulik *et al* (1979). In the phenytoin-treated embryos, the mesenchymal cellular processes, which form a dense meshwork that interact with the epithelial basement membrane, are underdeveloped or are absent. The hypothesized effect is secondary to interference of the drug with oxidative metabolism and ATP production by the cells. Mackler *et al* (1975) proposed that PHT, or the arene oxides from PHT, inactivate oxidative enzymes such as DPNH oxidase, and thus interfere with oxidative metabolism.

Genetically determined differences in metabolism of phenytoin may explain why Fetal Hydantoin Syndrome only occurs in a small portion of human foetuses who are exposed to the drug (Strickler *et al*, 1985). Inbred and congenic strains of mice have been studied for susceptibility to phenytoin induced cleft lip (Goldman *et al*, 1983). The role of genes linked to the H-2 complex on

chromosome 17 has been confirmed.

In a study, where Hicks *et al* (1983) collected mouse embryos from phenytoin treated mothers, it was found that the DNA and protein synthesis were altered by this drug. DNA synthesis in these tissue was only 26% of the control group value, but the protein synthesis was 2.6 times that of the control primary palate. These effects of phenytoin are not limited to primary palates for similar changes in DNA and protein synthesis also occur in embryonic limb buds.

IV. Oxygen

Hypoxia-induced cleft lip in CL/Fr mice has been reported by Millicovsky and Johnston (1981). The spontaneous clefting rate of 36% in CL/Fr mice was shown to increase to approximately 90% when pregnant mothers were exposed to hypoxia, (10% O_2), and to decrease to 13% when they were exposed to hyperoxia, (50% O_2), during the critical time of primary palate development. In a morphological study (Bronsky *et al*, 1986), cellular debris was present in hypoxic embryos at stages prior to primary palate fusion and absent in comparable stage normoxia embryos. It was suggested that this cellular debris was associated with the retardation of placodal invagination and was primarily responsible for the increased incidence of CL(P). In addition, it was hypothesized that placode invagination involves energy-dependent actin-myosin interactions, and is thus sensitive to the ATP-reducing effects of hypoxia.

2. Rationale

In the past, many studies have looked at human primary palate and cleft lip formation (Stark, 1954; Warbrick, 1960; Tondury, 1964; Anderson and Matthiessen, 1967; Vermeij-Keers, 1972; Hinrichsen, 1985; Diewert and Shiota, 1990). These studies utilized several human embryos at relevant stages, but too few embryos are available to reach any definite conclusion. Early facial development and morphology of human and mouse embryos are very similar and of comparable size at the time of lip formation (Trasler, 1968). The mouse model has been studied for cleft lip morphologically (Reed, 1933; Trasler, 1968; Juriloff and Trasler,1976; Trasler and Machado, 1979; Millicovsky *et al*, 1982; Ciriani and Diewert, 1985), teratogenetically (Sulik *et al*, 1979; Juriloff, 1981; Eto *et al*, 1981; Trasler and Leong, 1982; Trasler and Ohannessian, 1983; Juriloff and Harris, 1985; Bronsky *et al*, 1986) and genetically (Reed, 1936; Davidson *et al*, 1969; Bornstein *et al*, 1970; Juriloff, 1980; Juriloff and Fraser, 1980; Biddle and Fraser, 1986; Juriloff, 1986). However, there have been no quantitative studies looking at internal structures of normal and abnormal lip formation in mice with different genetic backgrounds.

The purpose of this study was to give insight into the etiology of cleft lip by measuring and analyzing internal structures of the forming primary palate in two noncleft, and three cleft lip strains of mice. The rationale behind the use of two noncleft lip strains was to compare the effect of different genetic background without the genetic cleft lip liability on primary palatal structures. The three cleft lip strains are investigated to determine the effects of different genetic backgrounds,

with different cleft lip and resorption rates, on the internal structures of primary palate.

The first specific aim was to collect embryos of three cleft lip strains at day 13 hour 12, and determine the cleft lip frequency and resorption rates of each strain. The rates were then compared among three cleft lip strains using statistical analysis. The uterine site effect on the cleft lip and resorption rate was also compared statistically.

The second specific aim was to determine the stage of body development and the chronological age at which primary palatogenesis takes place in these five strains. The chronological ages of these strains was used only to collect embryos at similar tail somite stages. The analysis of primary palate internal structure in the noncleft lip and cleft lip strains is based on developmental tail somite number which is more representative of developmental stage than is chronological age.

The third specific aim was to delineate and quantitatively compare progressive phases of primary palate development by studying the internal anatomical structures which were suggested as essential for successful upper lip formation. These phases are epithelial fusion forming nasal fin, interruption of the nasal fin with mesenchyme, mesenchymal enlargement, and cleavage of nasal fin forming the oronasal membrane and primary choana.

At an early stage of primary palate formation involving fusion of facial prominence epithelia and forward growth of the maxillary prominence, one noncleft lip and one cleft lip strain of mice were studied. This was done to

understand the possible factors which may contribute to cleft lip at the stage before the mesenchyme connects the medial and lateral walls of the primary palate. The next stage of primary palate formation with the mesenchyme replacing the epithelial nasal fin was investigated using two noncleft lip, and three cleft lip strains. An analysis of covariance comparing area of primary palate and mesenchymal component was used. The partial least squares analysis was used to determine the best predictor of primary palate formation. The primary choana dorsal to the primary palate forms at a later stage during primary palate formation when the oronasal membrane retracts, and a respiratory passage opens from the nostril through the primary choana to the pharynx. The timing of this event was investigated and compared in one noncleft lip strain and one cleft lip strain.

Finally, a multifactorial threshold model was suggested. Within the model, the genetic cleft lip liability is proposed to be influenced by the biological traits of primary palate formation. At the threshold, a critical amount of mesenchyme must be present for normal primary palate formation to occur. Unfavorable growth of any biological traits brings the period of mesenchymal formation closer to the threshold resulting in an occurrence of cleft lip. This threshold may be affected by the timing of primary choana formation.

MATERIALS AND METHODS

A. Cleft lip frequency and resorption rate.

I. Embryo collection

The cleft lip strains studied were A/J obtained from Jackson Laboratory, Bar Harbor, Maine, A/WySn obtained from Jackson Laboratory, Bar Harbor, Maine, and CL/Fr (developed in the laboratory of Dr. Fraser in McGill University). The strain of CL/Fr was imported to our laboratory in 1985. Mice were maintained on a diet of Purina mouse chow and filtered water and were housed with a 12 hr light cycle from 7 AM to 7 PM. Three or four adult females were caged overnight with a male and were examined in the morning for the presence of a vaginal plug. It was assumed that ovulation took place at midnight, therefore 9 AM of the day the plug was found was designated as day 0 hour 9 of gestation (Snell *et al*, 1940). Ten litters of A/J, eight litters of A/WySn and ten litters of CL/Fr were collected at day 13 hour 12. The embryos were dissected and numbered by the three digit system: the first digit indicated the right or the left site in the uterine horn; the right side was indicated as one and the left side was indicated as two; the second digit indicated the sequential order of the embryos from sites nearest the ovary to the cervix, and the third digit indicated the sequential order of the embryos from the sites nearest the cervix to the ovary; both the second and third digits were counted by numbers starting with one. No distinction was made as to the extent and form of the malformation. Thus, whether the clefting was complete or incomplete, unilateral or bilateral, the malformations were called cleft lip. Resorptions were defined as all dead embryos up to day 13 hour 12 of

development. Litters of less than 5 live embryos were discarded.

II. Data analysis

The frequency of resorptions among implantations and frequency of cleft lip among embryos in each litter were transformed to their Freeman-Tukey arc sine values (Mosteller and Youtz, 1961). One-way analysis of variance was applied to the transformed data. Effects of site of uterine implantation (ovarian or nonovarian) were tested in 2 X 2 Chi square tests for each resorption among implants and cleft lip among embryos.

B. Stages of primary palate development.

I. Embryo collection

Five strains of mice were used in this study: C57BL/6J (Charles River Canada Inc., Montreal), BALB/cByJ (Jackson Laboratory, Bar Harbor, Maine), and cleft lip strains, A/J, A/WySn and CL/Fr. Pregnant females were sacrificed at various times from day 10 hour 14 onwards. The uteri were removed from the animals and embryos were dissected and numbered using the three digit numbering system described above. The embryos were then fixed in Bouin's solution for at least 24 hours. Embryos were weighed and tail somite stage number, calculated by counting the number of pairs of somites from the hind limb to the last somite pair at the end of the tail, was recorded. Embryonic heads were then removed from the body with a No. 15 surgical blade and were photographed while immersed in 70 percent ethanol under standard conditions of lighting and magnification in frontal and ventral profile. The embryonic heads were processed

in an autotechnicon, embedded in paraffin, serially sectioned at 7 μm thickness in the frontal plane. Slides were then put in a 50 °C oven, then stained with hematoxylin and eosin or Periodic acid Schiff.

II. Measurements

a. Nasal fin

The nasal fin is the fused epithelia between medial nasal prominence, lateral nasal prominence and/or maxillary prominence and is connected to the nasal groove at the upper portion and oral ectoderm at the bottom portion. Calculation of the depth of the nasal fin in the serial anteroposterior sections was started from the first section containing fusion of the three facial prominences and ended at a point where the nasal fin disintegrated and preoptic mesenchyme appeared. A microruler of 100 grids (each grid equals 9.8 μm) in the Nikon microscope was used to measure the height of the fused epithelia (nasal fin) which spanned from the top of the nasal epithelium to the bottom of the oral ectoderm. The total area of the nasal fin was the summation of the areas from the serial sections calculated by multiplying the measured height by the tissue section thickness.

b. Mesenchymal component

The mesenchymal component is the mesenchymal tissue replacing the nasal fin epithelium. The mesenchyme grows in about 12 hours after epithelial nasal fin formation begins. The same micrometer and magnification as above was used to measure the height of the mesenchymal component, which spanned from the bottom of the nasal epithelium to the top of the oral ectoderm. At the

stage where the mesenchymal tissue replaces the nasal fin, the region was no longer called the nasal fin but the primary palate. The primary palate area is the summation of the areas from each section calculated by multiplying the height of the nasal epithelium at the top, the mesenchymal component in the middle, and the oral ectoderm at the bottom by the tissue section thickness. A similar calculation is done for the area of the mesenchymal component using the height of the mesenchyme only.

c. Position of the maxillary prominence

The maxillary prominence is an anatomical structure lateral to the lateral nasal prominence and superficially distinguished from the nasal prominence by the naso-maxillary groove. The maxillary prominence and lateral nasal prominence grow in a frontomedial direction and become fused with the medial nasal prominence. The position of the maxillary prominence was determined by counting the sections from the frontomedial end of the maxillary prominence as defined by the naso-maxillary groove to the dorsal end of the nasal fin. The number of sections was multiplied by 7 μm to determine the measurement of the position of the maxillary prominence.

III. Data analysis

a. Analysis of covariance

The analysis of covariance makes use of the concepts of both analysis of variance and regression. In a one-way classification, the typical analysis of variance model for the value Y_{ij} of the jth observation in the ith class is

$$Y_{ij} = \alpha_i + e_{ij}$$

where the α_i represent the population mean of the classes and the e_{ij} are the residuals. But suppose that on each unit we have also measured another variable X_{ij} that is linearly related to Y_{ij} . It is natural to set up the model,

$$Y_{ij} = \alpha_i + \beta (X_{ij} - \overline{X}_{..}) + \varepsilon_{ij} \quad [1]$$

where β is the regression coefficient of Y on X. This model is typical for the analysis of covariance. If X and Y are closely related, we may expect this model to fit the Y_{ij} values better than the original analysis of variance model. That is, the residuals ε_{ij} should be in general, smaller than the e_{ij}. In this study, a simple example of the use of covariance in randomized experiments is demonstrated. With a completely randomized design, the data form a one-way classification with the strains being the classes.

i. Testing adjusted treatment means

Table 1 gives the analysis of covariance for a randomized complete-block design and, at the same time, illustrates the general procedure. The general procedure requires all three sums of products for treatments and for error after adjustment for all other sources of variation included in the model. For a completely randomized design, there would be no block sums of products. From

---

[1] $\overline{X}_{..} = \dfrac{X_{..}}{n}$

$X_{..} = \Sigma X_i$

Source	df	Sums of products of			df	Adjusted Σy^2	MS
		X,X	X,Y	Y,Y			
Total	rt - 1	Σx^2	Σxy	Σy^2			
Blocks	r - 1	Rxx	Rxy	Ryy			
Treatments	t - 1	Txx	Txy	Tyy			
Error	(r - 1)(t - 1)	Exx	Exy	Eyy	(r - 1)(t - 1) - 1	$Eyy - \frac{(Exy)^2}{Exx}$	$S^2_{y \cdot x}$
Treatments + error	r(t - 1)	Sxx	Sxy	Syy	r(t - 1) - 1	$Syy - \frac{(Sxy)^2}{Sxx}$	
Treatments adjusted					t - 1	$\left(Syy - \frac{(Sxy)^2}{Sxx}\right) - \left(Eyy - \frac{(Exy)^2}{Exx}\right)$	

Table 1. Analysis of covariance for a randomized complete-blocked design (From Steel and Torrie, 1980).

the treatments and error line, a line for treatments plus error is obtained by addition. The sums of squares E_{yy} and S_{yy} are adjusted by subtracting the contributions due to linear regression. The difference between these adjusted sums of squares is the sum of squares for testing adjusted treatment means. To test the mean square for adjusted treatments, the appropriate error mean square is $S^2_{Y.X}$. Notice that the lines for treatments and error are essential from which the test of adjusted treatment means is constructed.

The calculation of sums of products for the randomized-block design is as follows (Steel and Torrie, 1980)

$$\sum x^2 = \sum X_{ij}^2 - \frac{X_{..}^2}{rt}$$

$$\sum y^2 = \sum Y_{ij}^2 - \frac{Y_{..}^2}{rt}$$

$$\sum xy = \sum X_{ij}Y_{ij} - \frac{X_{..}Y_{..}}{rt}$$

Sum of products for blocks are:

$$R_{xx} = \frac{\sum_j X_{\cdot j}^2}{t} - \frac{X_{\cdot\cdot}^2}{rt}$$

$$R_{yy} = \frac{\sum_j Y_{\cdot j}^2}{t} - \frac{Y_{\cdot\cdot}^2}{rt}$$

$$R_{xy} = \frac{\sum_j X_{\cdot j} Y_{\cdot j}}{t} - \frac{X_{\cdot\cdot} Y_{\cdot\cdot}}{rt} \quad [2]$$

Sums of products for treatments are:

$$T_{xx} = \frac{\sum_i X_{i\cdot}^2}{r} - \frac{X_{\cdot\cdot}^2}{rt}$$

$$T_{yy} = \frac{\sum_i Y_{i\cdot}^2}{r} - \frac{Y_{\cdot\cdot}^2}{rt}$$

$$T_{xy} = \frac{\sum_i X_{i\cdot} Y_{i\cdot}}{r} - \frac{X_{\cdot\cdot} Y_{\cdot\cdot}}{rt} \quad [3]$$

Sums of products for error are found by subtraction and are:

---

[2] $X_{\cdot j}$: the dot indicates that all observations for the *jth* block have been added to give this total.

[3] $X_{i\cdot}$: the dot indicates that all observations for the *ith* treatment have been added to give total.

$$E_{xx} = \Sigma x^2 - R_{xx} - T_{xx}$$

$$E_{yy} = \Sigma y^2 - R_{yy} - T_{yy}$$

$$E_{xy} = \Sigma xy - R_{xy} - T_{xy}$$

To test the hypothesis of no differences among treatment means for Y adjusted for the regression of Y on X,

$$F = \frac{MS\,(\text{adjusted treatments means})}{s^2_{Y \cdot X}}$$

$$s^2_{Y \cdot X} = \frac{E_{yy} - \dfrac{(E_{xy})^2}{E_{xx}}}{(r-1)(t-1)-1}$$

$$MS = \frac{\left[S_{yy} - \dfrac{(S_{xy})^2}{S_{xx}}\right] - \left[E_{yy} - \dfrac{(E_{xy})^2}{E_{xx}}\right]}{t-1}$$

"The one-tailed *F* test with 1 and *n* degrees of freedom corresponds to the two-tailed *t* test with *n* degrees of freedom. This *t* test does not specify the direction of the difference between two treatment means for the alternative hypothesis; thus it is like the one-tailed *F* test which specifies which mean square is to be the larger

as the result of differences of unspecified direction between treatments. These tests can be shown to be algebraically equivalent; in particular $t^2 = F$. The relation is shown graphically in Figure 2. Small numerical values of *t*, when squared, become small values of *F*, positive quantities. Large numerical values of *t*, when squared, become large values of *F* (Steel and Torrie, 1980)."

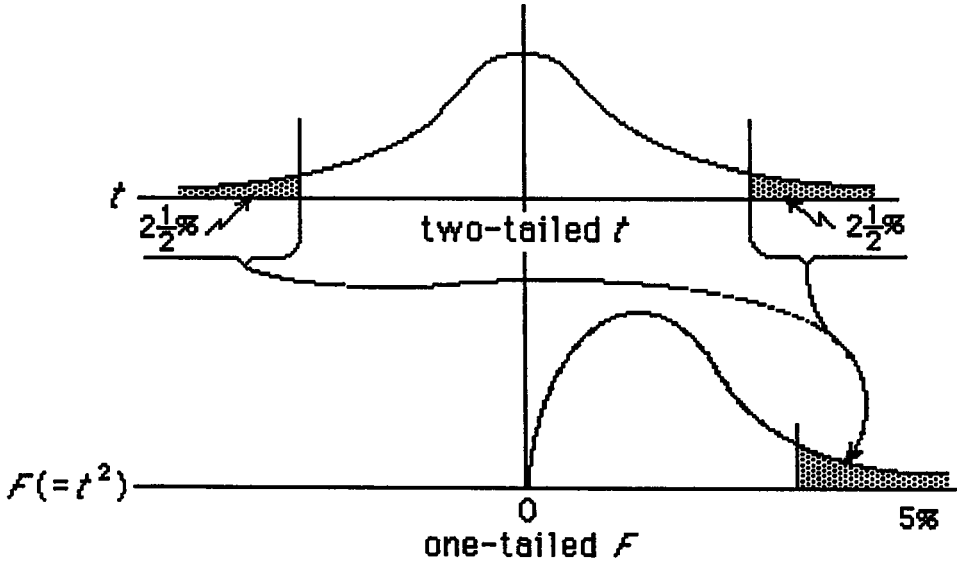


Fig. 2. Relation between two-tailed t and one-tailed F (curves are only approximate) (From Steel and Torrie, 1980).

ii. Homogeneity of regression coefficients

Where the experimental design is a completely random one, the regression of Y on X can be computed for each treatment. In this case, the usual

assumption of homogeneity of the regression coefficients can be posed as a null hypothesis and tested by an appropriate *F* test in an analysis of covariance (Sokal and Rohlf,1969).

The procedure that follows is an *F* test for difference between two regression coefficients:

$$F = \frac{(b_1 - b_2)^2}{\frac{\sum x_1^2 + \sum x_2^2}{(\sum x_1^2)(\sum x_2^2)} \cdot \overline{s}^2_{y \cdot x}}$$

where $\overline{s}^2_{y \cdot x}$ is the weighted average. For two groups we can write its formula as (Sokal and Rohlf,1969).

$$\frac{\sum y_1^2 - \frac{(\sum xy)_1^2}{\sum x_1^2} + \sum y_2^2 - \frac{(\sum xy)_2^2}{\sum x_2^2}}{n_1 + n_2 - 4}$$

Since there is a single degree of freedom in the numerator, $t = \sqrt{F}$.

b. Partial Least Squares (PLS) analysis

PLS is a hybrid regression analysis and factor analysis which has recently been applied to diverse scaling problems in the natural and social

sciences (Bookstein, 1982; 1986; Wold, 1982). It is a method of data reduction. An investigator has collected two "blocks" of indicators and wishes to summarize the predictive interrelations among the set of these two blocks considered together. Each indicator was intended to tap some aspect of a construct underlying its entire block. (For example, the construct for the general body item is "develop", and for the primary plate parameters is "palate".) Yet our interest is not so much in that underlying construct (its factors, its reliability, etc.) as in its correlations with other construct or constructs of the full data set, which are also measured indirectly via their own indicators. Regardless of the correlations among primary palate parameters, different parameters are sensitive to effects of body size to different extents. We therefore wish to scale the items of each block of indicators to best explain the cross-block relationships (correlations). These mutually scaled scores are the *latent variables* as they are constructed by PLS for two blocks at the same time.

Appendix 3 displays the ordinary correlation coefficients between each of three measures of development indices and each of the five primary palate parameters from five mouse strains. This array displays a clear pattern of signs: the correlations of all of the developmental indices with respect to all of the primary palate parameters are positive. There appears to be a stable positive correlation between general body size development as measured in this battery, and primary palate growth as assessed by the parameters of five mouse strains.

In combining different estimates of the same quantity that vary in precision, it is standard to weight the contribution of each in proportion to its

precision, so that the more precise estimates are given more weight in forming the average. Likewise, in attempting to construct a net score (latent variables) for development that is to correlate with palate, we should weight the development in proportion to their correlation with the sum of the primary palate parameters. This is what a partial least squares does.

Such a two-block analysis is typically diagrammed as shown in Figure 3. Observed variables are indicated by squares and latent variables by circles. The single line between the two latent variables indicates our intention to explain the pattern of correlations between observations of different blocks in terms of a single pair of latent variables. We are not attempting to explain the correlations among indicators of the same block; instead we are determining the linear combinations of the indicators in each block which are predictive of items in the opposite block.

The prescription just given for the latent variables of interest can be expressed simply in algebra as follows (Sampson *et al*, 1989). The Development latent variable (LV) scores are written as

$$LV_A = \alpha_1 A_1 + \cdots + \alpha_3 A_3 = \sum_{i=1}^{3} \alpha_i A_i$$

where $A_1, \ldots, A_3$ are the three developmental indicators, scaled to have variance one, and $\alpha_1, \ldots, \alpha_3$ are three positive weights to be computed. The α' s are to be proportional to the correlations of the A's with a similarly weighted sum

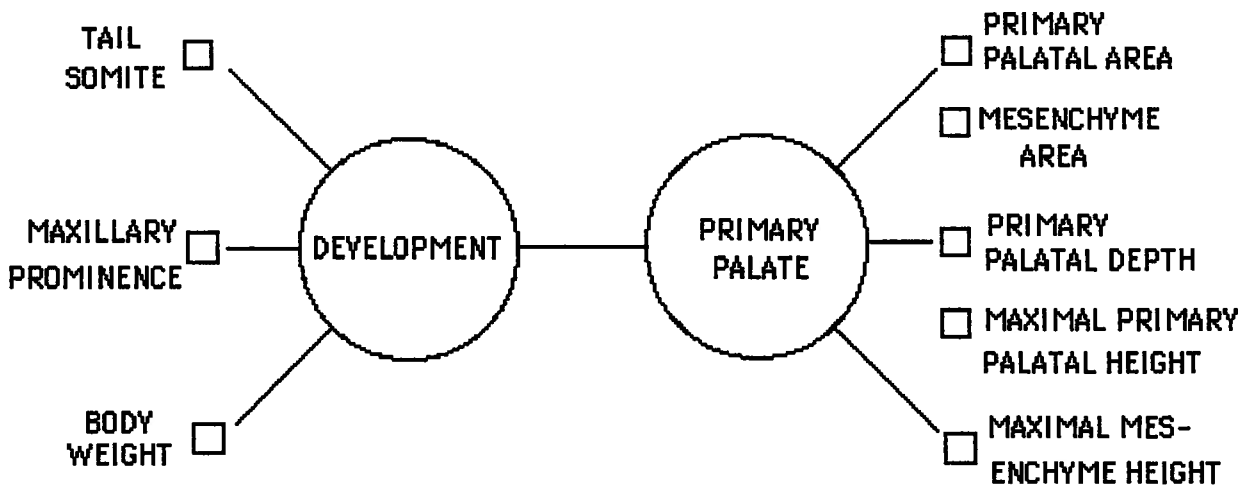


Fig. 3. Diagram for a two-block latent variable model relating 3 indicators of development to 5 parameters from the primary palate.

$$LV_B = \beta_1 B_1 + \cdots + \beta_5 B_5 = \sum_{j=1}^{5} \beta_j B_j$$

of the five primary palate parameters. That is,

$$\begin{aligned} \alpha_i &\propto \text{corr} \ (A_i, \ \Sigma \beta_j B_j) \\ &\propto \text{cov} \ (A_i, \ \Sigma \beta_j B_j) \\ &= \sum_{j=1}^{5} r_{ij} \beta_j \end{aligned} \qquad (1)$$

where r_{ij} is the correlation of development item i and palate item j, the (i, j) element of the matrix R_{AB} of correlations given in Appendix 3. Note that all of the (scaled) palate variables are treated equally in determining the coefficient α_i of the development variable A_i. The weights β_j of the palate variables are similarly required to satisfy

$$\beta_j \propto \sum_{i=1}^{3} \alpha_i r_{ij} \qquad (2)$$

For convenience, we scale the weights so that $\Sigma \alpha_i^2 = \Sigma \beta_j^2 = 1$.

"Thus each coefficient, α_i or β_j is computed as a simple covariance, or regression coefficient (salience), corresponding to an optimal least squares prediction using part of the data. For this reason we call the linear combination $\Sigma \alpha_i A_i$ the " net partial predictor " (NPP) of LV_B, denoted NPP ($LV_B \mid A_1, \ldots, A_3$). These NPP's stand in contrast to multiple regression predictors. Estimates of the

coefficients are typically computed using an iterative algorithm, alternately updating estimates of the α_i from equation (1), and then the β_j from equation (2). Such an iterative procedure constitutes a Partial Least Squares algorithm (Wold, 1982). The algorithm may be summarized conveniently as follows:

0. Initialize $LV_A \propto (A_1 + \ldots + A_3)$; that is, set $\alpha_1 = \ldots = \alpha_3 = 1 / \sqrt{3}$.

1. Compute the linear combination of 5 primary palate parameters as $LV_B = \sum \beta_j B_j = NPP (LV_A \mid B_1, \ldots, B_5)$, where each β_j is defined as in equation (2) with $\sum \beta_j^2 = 1$.

2. Compute the linear combination of 3 developmental scores as $LV_A = \sum \alpha_i A_i = NPP (LV_B \mid A_1, \ldots, A_3)$, where each α_i is defined as in equation (1) with $\sum \alpha_i^2 = 1$.

3. Return to 1 and iterate until LV_A and LV_B fail to change to some preset tolerance." (Sampson *et al*, 1989).

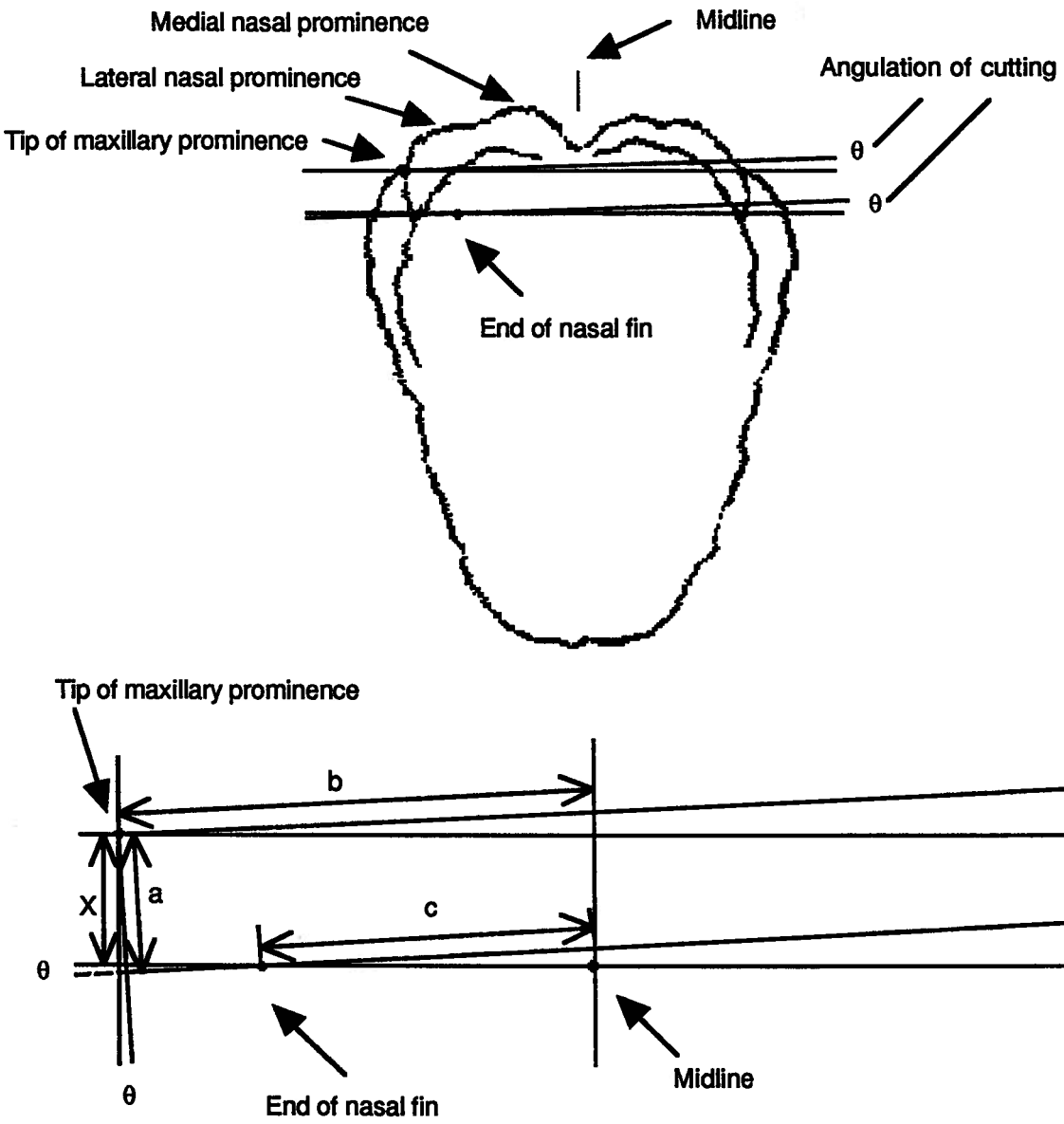
A computer program which implements this algorithm is from Dr. F. Bookstein.

For example, the differences in the primary palate parameters, which include the primary palate area and the mesenchymal bridge area, among five mouse strains are analyzed by analysis of covariance, with the tail somite as covariable to test the specific strain effects. In addition, the latent variables of tail somite, body weight and maxillary prominence depth based on partial least square analysis, were also used as the covariable in the study of strain specific

effects. The latent variable of tail somite, body weight, with the entailments of maxillary prominence growth were operated by normalizing each indicator by assigning 0 as the mean and 1 as the standard deviation. Normalized indicators were then multiplied by each simple covariance generated from partial least square analysis called "salience". Summation of each multiplied value is the latent variable of each embryo. Such strain effects on primary palate growth were more precisely expressed as residuals because the growth of maxillary prominence is linearly related to the regional growth of primary palate parameters.

IV. Error of measurement

The maxillary prominences were tested for strain specific effects among five strains by analysis of covariance using tail somite as the covariable. The errors of measurement of the position of maxillary prominence were determined from both superior and sagittal view. As the angulation of the cutting may deviate to right or left side of the head in superior view as shown in Figure 4, the real position of the maxillary prominence can be expressed by the measured position of the maxillary prominence and an angulation of θ. The angulation θ can be calculated from the bimaxillary distance and difference between right and left depth of the maxillary prominence as they are cut in different levels. From the angulation and the measured position of the maxillary prominence, the true depth can be calculated as in the equation of Figure 4. The error from angulation of section is then generated by deducting the true depth from the measured depth.



$$X = (a - (b - c - (a \times \tan\theta)) \times \tan\theta) \times \cos\theta$$

a = measured depth of the maxillary prominence.

b = half of the measured bimaxillary width.

c = measured distance from the end of the nasal fin to midline.

Fig. 4. Error of measurement for the depth of the maxillary prominence from the superior view. The cutting deviates from the perpendicular line to the midline with an angle θ. This angle can be calculated from the bimaxillary width and the difference between left and right depth of maxillary prominences. From the angulation and the equation shown in this graph, the true depth of maxillary prominence can be generated.

Then the results of angle error were compared with standard deviation of position of right maxillary prominence. In BALB/cByJ, the errors from angulation, which averaged two degrees, are 5.05 μm at 13 tail somites and 10.87 μm at 16 tail somites, while the respective standard deviations were 22.13 μm and 21.12 μm. For A/J, the same errors of 13 and 16 tail somites are 6.54 μm and 8.70 μm, and standard deviations of 13 and 16 tail somites were 21.76 μm and 21.80 μm. As the errors fall into the standard deviation's range, it is not necessary to correct the data for error in angulation.

From the sagittal view, as the angulation of the cutting may deviate forward or backward as shown in Figure 5, the position of the maxillary prominence can be varied by the angulation of tipping as well. From the calculation by using the mandibular prominence as reference, the angles of tipping can be calculated (Fig. 5). If the position of maxillary prominence, using the cutting through both tips of maxillary and mandibular prominence as reference, is considered as the true position, it can be calculated from measured depth and angulation shown in Figure 5. The error from angulation of section is then generated by deducting the true depth from the measured depth. The results of angle error were compared with the standard deviation of the position of the right maxillary prominence. In BALB/cByJ, the errors from angulation, which averaged twenty degrees, are 10.08 μm at 13 tail somites and 14.58 μm at 16 tail somites, while the respective standard deviations were 22.13 μm and 21.12 μm. For A/J, the same errors for 13 and 16 tail somites are 8.74 μm and 13.08 μm, and standard deviations for 13 and 16 tail somites were 21.76 μm and 21.80 μm. As

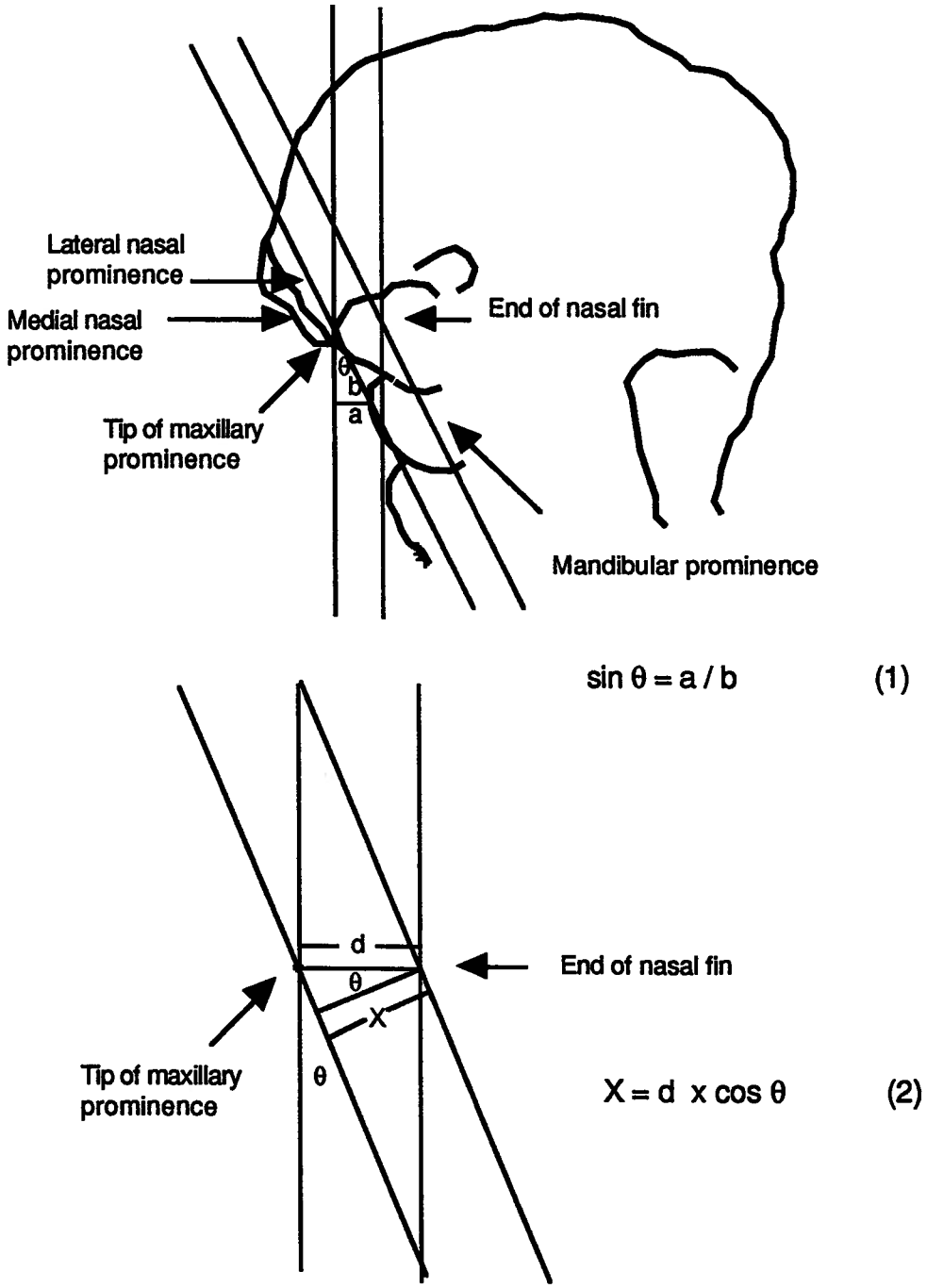


Fig. 5. Error of measurement of the depth of the maxillary prominence from the sagittal view. The cuttings may deviate forward or backward. The depth of the maxillary prominence are varied by the angulation θ. This angulation can be calculated from the distance between tip of maxillary and mandibular prominence and the depth between two cuttings (equation 1). From the angulation and the equation 2, the true depth of maxillary prominence using the cutting through both the tip of maxillary and mandibular prominence as reference can be generated.

the errors also fall into the standard deviation's range, correction of the data for error in angulation is not necessary.

C. Stages of primary choana formation

Embryos of C57BL/6J and CL/Fr were also collected for studying the formation of the primary choana. From the serial frontal sections, the structure of the oronasal membrane was identified and the cavitation of the oronasal membrane to form the primary choana was studied in these two strains. The tail somites of embryos with primary choanae formation were used as reference for the comparison. Effects of strains on time of primary choana formation were tested by 2 X 2 Chi square tests.

RESULTS

A. Cleft lip frequency and resorption rate

I. Strain effect

The frequency of cleft lip from 10 litters of A/J is 4.0% (Table 2). Much higher cleft lip frequencies occurred in A/WySn and CL/Fr, 22.5% and 23.9%. The arc sine transformed value was significantly smaller in A/J but did not differ between A/WySn and CL/Fr (Table 2). Thus the cleft lip frequency falls into two groups; A/J (low cleft lip frequency) versus A/WySn and CL/Fr (high cleft lip frequency). Embryonic resorption rates are also shown in Table 2. Day 13 hr 12 resorption rate was higher in A/J (18%) and lower in A/WySn and CL/Fr, 5.3% and 12.2%. The arc sine transformed values differed significantly between A/J and A/WySn.

II. Uterine site effect

The relationship of cleft lip and resorption frequency and uterine position were analyzed by dividing each uterine horn into ovarian site and other sites. The malformation and resorption frequencies in them were compared (Table 3). The cleft lip frequency was higher in ovarian sites than other sites in all three strains; however, for each strain, the difference analyzed by the Chi square test was not significant. Analysis of the segments of three strains pooled for independence by 2 X 2 tests showed that the frequency of cleft lip at the ovarian site (13/48 = 27.1%) was significantly different (Fisher's exact test, $p = 0.014$) from other sites (24/190 = 12.6%). However, the resorption rate at the ovarian site (7/55 = 12.7%)

Strain	No. of Litters	No. of Implantations	Mean number of implantations	Resorbed (%)	Mean arc sine(%) resorbed ± SE[1]	No. of embryos	% cleft lip embryos	Mean arc sine(%) CL ± SE[2]
A/J	10	117	11.7	18.0	26.37 ± 2.17[a]	96	4.0	13.78 ± 2.17[a]
A/WySn	8	75	9.4	5.3	15.27 ± 2.34[b]	71	22.5	29.80 ± 2.34[b]
CL/Fr	10	82	8.2	12.2	21.19 ± 2.50[ab]	72	23.6	30.59 ± 4.29[b]

Table 2. Cleft lip and resorption rate in embryos of day 13 hour 12 of three cleft lip strains.

[1] $F_{2, 25} = 5.30$; $p < 0.05$.

[2] $F_{2, 25} = 9.75$; $p < 0.001$.

[a,b] means sharing the same superscript do not differ ($p > 0.05$) by Duncan multiple-range test (Cody and Smith, 1987).

Strain	Number of fetuses		% cleft lip		Number of implants		% resorbed	
	Ovarian site	Other sites	Ovarian site	Other sites	Ovarian site	Other sites	Ovarian site	Other sites
A/J	16	80	12.5	2.5	20	97	20.0	17.5
A/WySn	15	56	33.3	19.6	16	59	6.3	4.0
CL/Fr	17	54	35.3	20.4	19	62	10.5	12.9
All pooled	48	190	27.1 [a]	12.6	55	218	12.7 [b]	12.8

Table 3. Effects of implantation site on frequency of cleft lip and resorption.

[a] % of cleft lip at the ovarian site is significantly different from other sites (Fisher's exact test, $p = 0.014$)
[b] % of resorption at the ovarian site is not different from other sites.

was not significantly different from other sites (28/218 = 12.8%).

B. The development of chronological age and tail somites of five strains during primary palate formation

Primary palate development in mice starts when the epithelia of the three facial prominences fuse together. This occurs at 8 tail somites in C57BL/6J and CL/Fr. The oronasal membrane breaks down to form the primary choana. This also happens at 18 tail somites in CL/Fr and C57BL/6J. Since the enlargement and elevation of the shelves of the secondary palate follows primary choanae formation (Tamarin, 1982), the primary choana is considered as an anatomical delineation for successful primary palate development. The five strains of mice studied have a distribution of chronological age and tail somite stage as shown in Figure 6.

A/J, A/WySn and CL/Fr have the same chronological age (day 11 hour 2 to day 11 hour 18) at the same interval of tail somites (8 to 18 tail somites). BALB/cByJ has the same chronological age as the three cleft lip strains at the same interval of tail somites. In contrast, at the same range of tail somites the chronological age of C57BL/6J is younger (from day 10 hour 17 to day 11 hour 11) than the above four strains.

C. Delineation of phases of primary palate development

From 8 to 12 tail somites, the early stage of primary palate development including the fusion of medial and lateral nasal epithelia to form the nasal fin and

Age	C57BL/6J	BALB/cByJ	A/J	A/WySn	CL/Fr
D10/17	8				
D10/18	9 10 11				
D10/19					
D10/20	9 9 9 10 10 10 10 11 11 12 12				
D10/21					
D10/22					
D10/23					
D11/0	12 12 12 12 12 12 13 13 13 13 13 13 14 14 14 14 14 14 15 15 15 16 16				
D11/1					
D11/2					8 8 8 10 10 10
D11/3					
D11/4					
D11/5	14 15 16 16 17 17 17 17 17 17 18 18 18 18 18 18				
D11/6	13 13 14 14 14 14 14 15 15 15 16 16 16 16 17 17 17 17				
D11/7					
D11/8	8 8 14 15 15 15 15 15 16 17 17 17			12 14 14 15 16 16 16	8 8 9 12 13
D11/9					
D11/10		10 11 12 12 13 14 15 15			
D11/11	16 17 18 18 18 18	11 14 14 14 14	14 15		
D11/12		11 12 12 13 14 14 15 15 15 15 15 15 15 16 17 17	13 13 14 15 15 15	12 12 15 15	9 9 9 11
D11/13		10 10 11 13 13 13 13 13 13 14 14 14 15 16 16	13 13 14 14 14 14 15 15 15 16 16 16 16 16		11 11 12 14 15 16 16 16 16 16 17 18 18
D11/14		14 15 16 16 17 20		15 15 17 17 18	8 12 13 13 14 14 15 15 16 16 16 17 17
D11/15				12 13 13 13 14 15	14 16 17
D11/16		15 17		13 14 14 17 17 17 17 17 19	13 14 15 16
D11/17			14 15 16	13 14 16 16	10 11 13 13 14 16 16 17
D11/18					14 15 15

Fig. 6. Tail somite distribution (8 to 18 tail somite stage) compared with primary palate development by strains. The specimen(s) of each chronological age is / are the embryo(s) collected and sectioned.

the forward growth of the maxillary prominence was studied in noncleft lip (C57BL/6J) and cleft lip (CL/Fr) strains. Depth of the nasal fin and position of the maxillary prominence of the noncleft lip strain was compared with the cleft lip strains. From 13 to 16 tail somites, replacement of the epithelial seam with mesenchyme ingrowth forming the mesenchymal component is anticipated during. Areas of the primary palate and the mesenchymal component, and position of the maxillary prominence, were compared among two noncleft lip (BALB/cByJ and C57BL/6J), and three cleft lip (A/J, A/WySn and CL/Fr) strains. Primary choana formation occurs from 18 to 20 tail somites, after the primary palate has definitely formed. Timing of primary choana formation of a noncleft lip strain (C57BL/6J) is compared with that of a cleft lip strain (CL/Fr).

I. Early primary palate development

At the stage of 8 tail somites, in CL/Fr and C57BL/6J, the lateral wall of the nasal pit is formed frontally by the lateral nasal prominence, while more caudally the maxillary prominence replaces the lateral nasal prominence. These two prominences are separated at the surface by the naso-maxillary groove (Fig. 7). The medial nasal prominence, which forms the medial boundary of the nasal pit, now touches both the lateral nasal prominence frontally and the maxillary prominence caudally (Fig. 7). The interposition of the epithelial plate or nasal fin maintains an epithelial continuity between the nasal cavity and the roof of the mouth (Fig. 8). The definition of the back end of the nasal fin is the separation of the nasal epithelium and the oral ectodermal epithelium by the preoptic

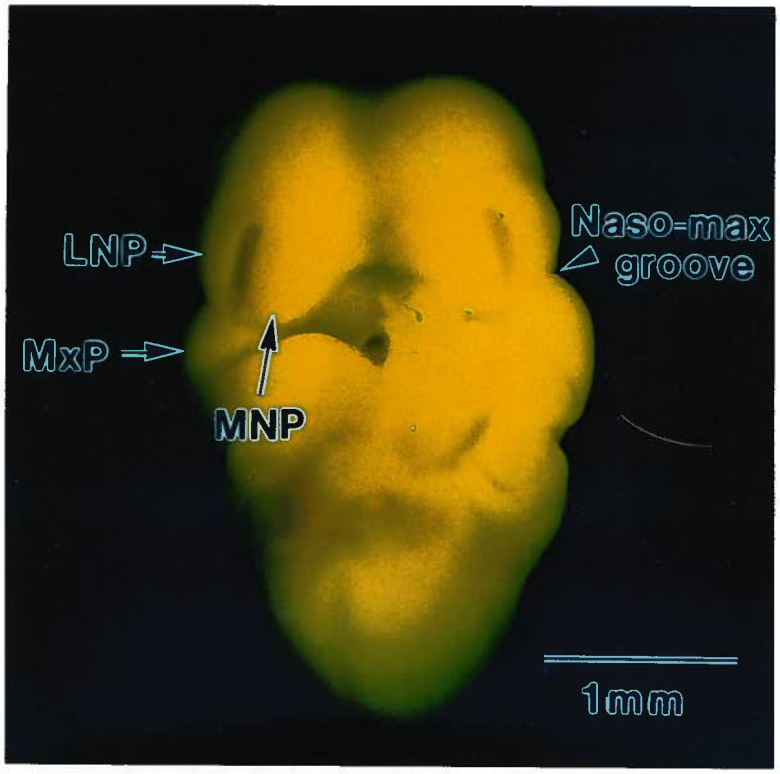


Fig. 7. Frontal view of C57BL/6J, day 10 hour 17, 8 tail somite stage. The lateral nasal prominence (LNP) and maxillary prominence (MxP) are separated by the naso-maxillary groove. The medial nasal prominence (MNP) which forms the medial boundary of the nasal pit touches both the lateral nasal prominence frontally and maxillary prominence caudally.

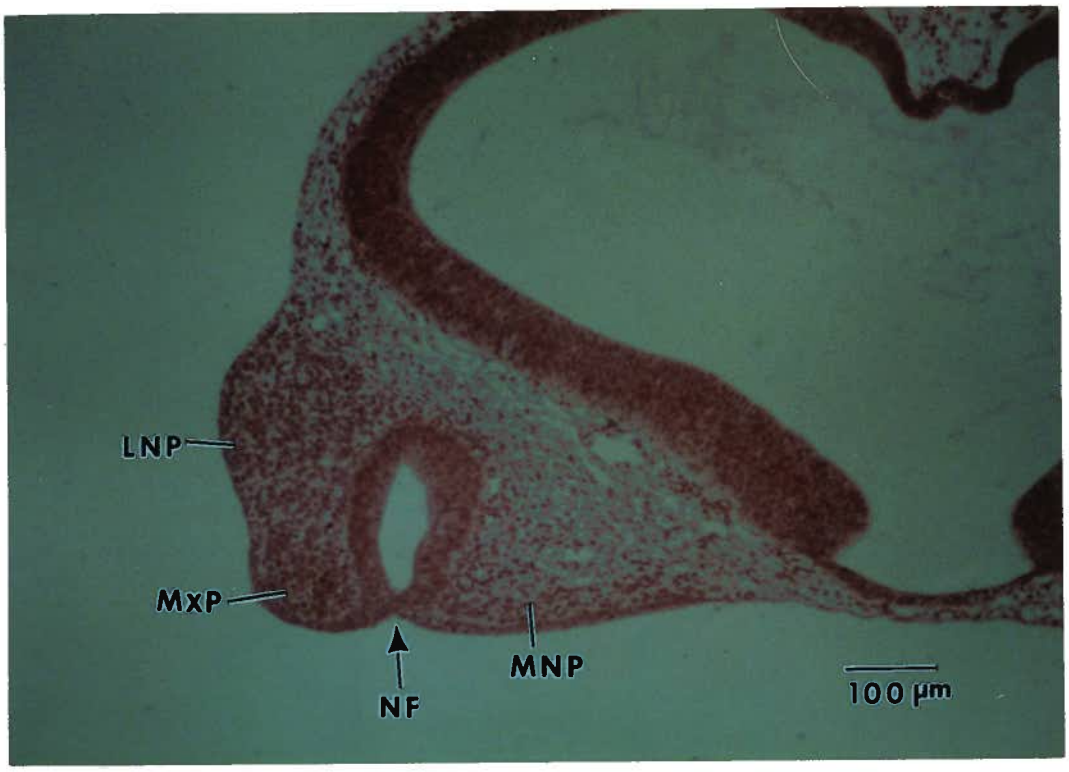


Fig. 8. Frontal section of C57BL/6J at the level of nasal fin, day 10 hour 17, 8 tail somite stage. The nasal fin (NF) which is composed of medial nasal prominence (MNP) at the medial side and lateral nasal (LNP) and maxillary prominence (MxP) at lateral side maintains the continuity between the nasal cavity and the roof of the mouth.

mesenchyme (Fig. 9).

The maxillary prominence in C57BL/6J mice extends frontally past the back end of the nasal fin with a positive position of the maxillary prominence relative to the end of the nasal fin at 8 tail somites (Table 4). In contrast the position of the maxillary prominence in CL/Fr is behind the end of the nasal fin at 8 tail somites. Thus the position of the maxillary prominence is defined negative (Table 4). Calculations of the number of sections show that the depth of nasal fin of C57BL/6J is not significantly larger than that of CL/Fr.

From the stage 9 to 12 tail somites, the chronological age of CL/Fr and C57BL/6J is approximately 11 days 8 hours to 11 days 14 hours and 10 days 20 hours to 11 days 0 hours (Fig. 6). The location of nasal fin, indicated on the surface by a groove, becomes deeper and marks the boundary between the medial nasal, lateral nasal and maxillary prominence in both the CL/Fr and C57BL/6J embryos (Fig. 10). Internally the proliferation of mesenchymal tissue inside the maxillary, lateral nasal and medial nasal prominence is very active in this stage. The nasal fin is not replaced by the mesenchymal cells in this stage (Fig. 11). By calculation of the nasal fin of CL/Fr frontocaudally the depth is smaller than C57BL/6J, although only left sides of 10 and 12 tail somites are significantly different (Table 4). The right and left maxillary prominences, which bulge on the surface as a prominent ridge, are still widely separated. In CL/Fr the maxillary prominence extends ventromedially over the end of the nasal fin with a positive position relative to the end of the nasal fin. The position of the maxillary prominence in CL/Fr is significantly less advanced than in C57BL/6J at 10 and 12

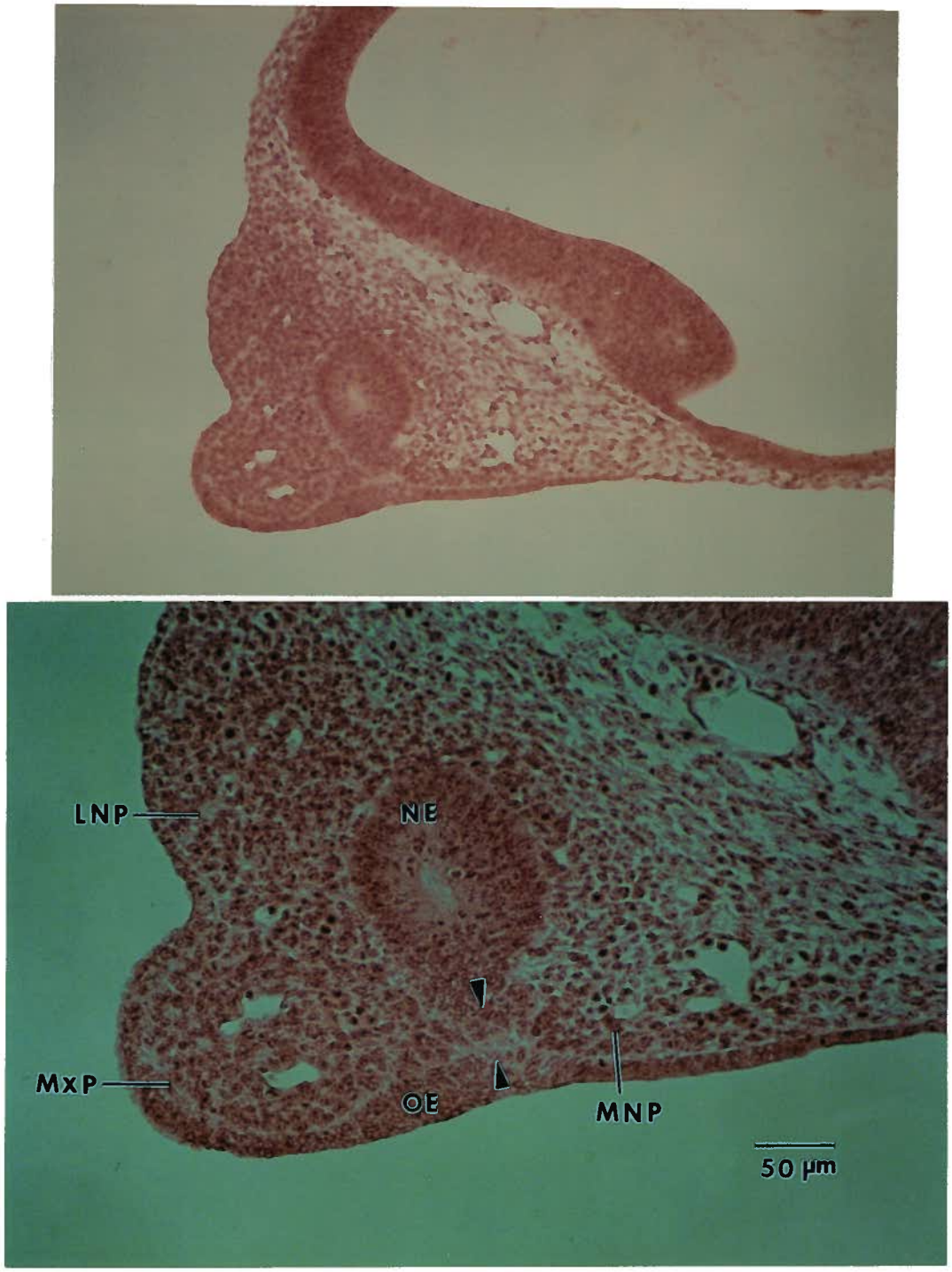


Fig. 9. Frontal section of C57BL/6J at the end of nasal fin, day 10 hour 17, 8 tail somite stage. The end of the nasal fin is shown in this photograph. The nasal epithelium (NE) and oral epithelium (OE) are separated by the preoptic mesenchyme (between the arrow heads). Thus the epithelial continuity does not exist between the nasal cavity and the roof of the mouth.

TS	Strain	(n)	Nasal Fin Depth (μm)		Position of maxillary. prom. (μm)	
			Right Mean±S.D.	Left Mean±S.D.	Right Mean±S.D.	Left Mean±S.D.
8	C57	3	39.7±8.0	46.7±14.6	28.0±18.5	37.3±4.0
	CL/Fr	6	30.8±6.3	32.2±3.8	-18.2±26.5	-12.6±18.1
9	C57	4	64.7±22.4	69.3±23.5	54.3±25.2	51.8±28.3
	CL/Fr	4	54.3±6.7	57.8±14.4	28.0±15.1	30.3±4.0
10	C57	5	146.4±34.9	137.2±28.3	102.2±25.5	91.0±16.4
	CL/Fr	6	99.2±60.8	80.5±32.2 *	45.5±21.1*	47.8±28.8*
11	C57	3	182.0±7.0	175.0±14.0	116.7±22.5	105.0±12.1
	CL/Fr	5	128.8±45.0	133.0±40.0	86.8±34.9	75.6±31.5
12	C57	5	221.2±13.7	226.2±21.2	142.8±15.3	148.4±15.2
	CL/Fr	4	127.8±78.2	89.3±61.9 *	75.3±14.5*	66.5±4.0 *

Table 4. Nasal fin depth and the position of the maxillary prominence of C57BL/6J and CL/Fr from 8 to 12 tail somite stage (TS). The position of the maxillary prominence depth of CL/Fr is negative relative to the end of the nasal fin at 8 TS because the position of the tip of the maxillary prominence is behind the end of the nasal fin. At 10 and 12 TS, the position of the maxillary prominence are significantly less advanced in CL/Fr than in C57BL/6J. The nasal fin depth is smaller in CL/Fr as well; however, only left sides of 10 and 12 TS are significantly different.

* $p < 0.05$ (between CL/Fr and C57).

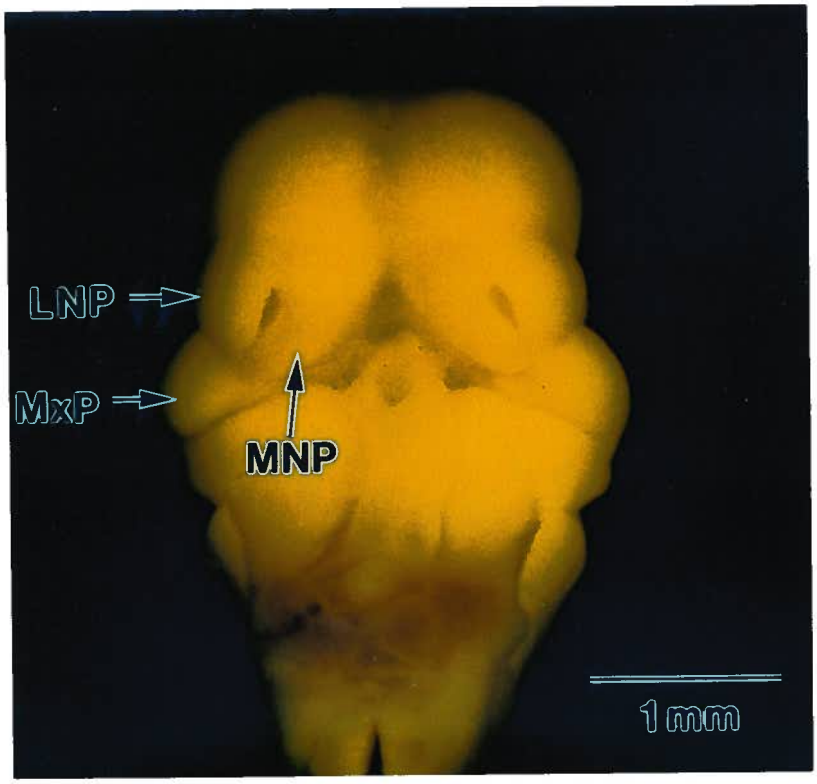


Fig. 10. Frontal view of C57BL/6J, day 11 hour 0, 12 tail somite stage. The boundary among the medial nasal prominence, lateral and maxillary prominences is marked by a deeper groove which indicates the position of the nasal fin.

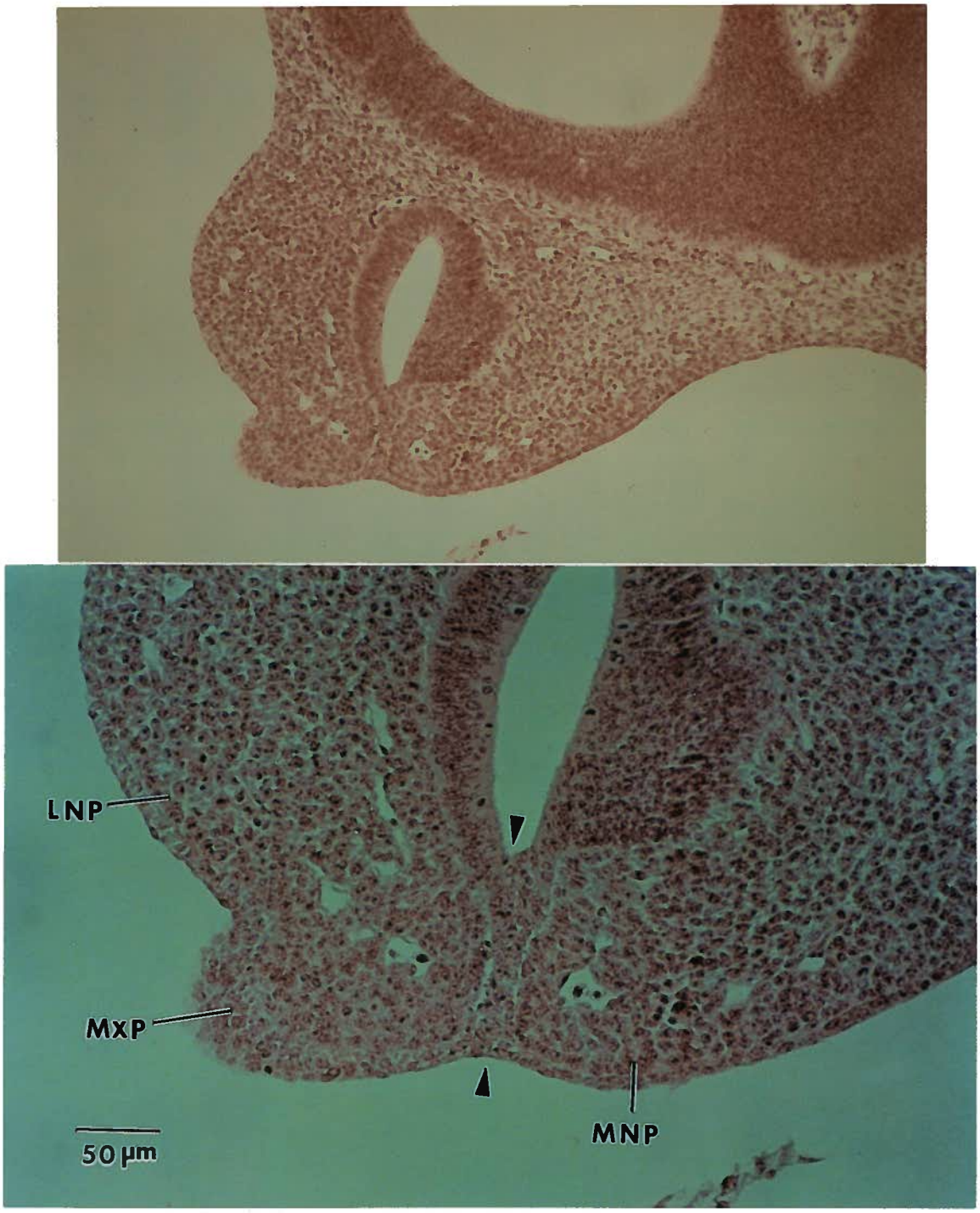


Fig. 11. Frontal section of C57BL/6J at the level of nasal fin, day 11 hour 0, 12 tail somite stage. The proliferation of mesenchymal tissue inside the maxillary, lateral and medial nasal prominence is very active. No nasal fin is replaced by mesenchymal tissue (between the arrow heads).

tail somites (Table 4).

Since the correlation between the depth of right and left side of nasal fin and the maxillary prominence is significant (R = 0.99 in nasal fin depth and 0.94 in maxillary prominence for C57BL/6J; R = 0.79 in nasal fin depth and 0.86 in maxillary prominence for CL/Fr), the right side was chosen randomly for testing the strain effect on the growth of the nasal fin and forward movement of the maxillary prominence. The analysis of covariance was used to analyze and compare the growth patterns of the nasal fin and the maxillary prominence. It combines the methods of regression and analysis of variance. The basic problem is to make inferences about group means of a dependent variable, such as depth of nasal fin or position of the maxillary prominence, that is measured in μm. Another variable, the tail somite number, called a covariable, is measured in whole units. Analysis of covariance makes use of information about depth of nasal fin or position of the maxillary prominence compared with tail somite development. Associated with the analysis of covariance is the study of differences in regression relationships among the groups by the test for heterogeneity of slopes. Covariance analysis test for differences in intercepts assuming a constant regression relationship among groups. The test for heterogeneity is a test for the validity of this assumption, and it tests whether or not the regression coefficients are constant over groups (Freund and Littell, 1981). Analysis of covariance of the nasal fin depth with the tail somite as a covariable showed that CL/Fr nasal fin depth increases slower than C57BL/6J's ($p < 0.05$) (Fig. 12, Table 5). However, covariance analysis of the position of right

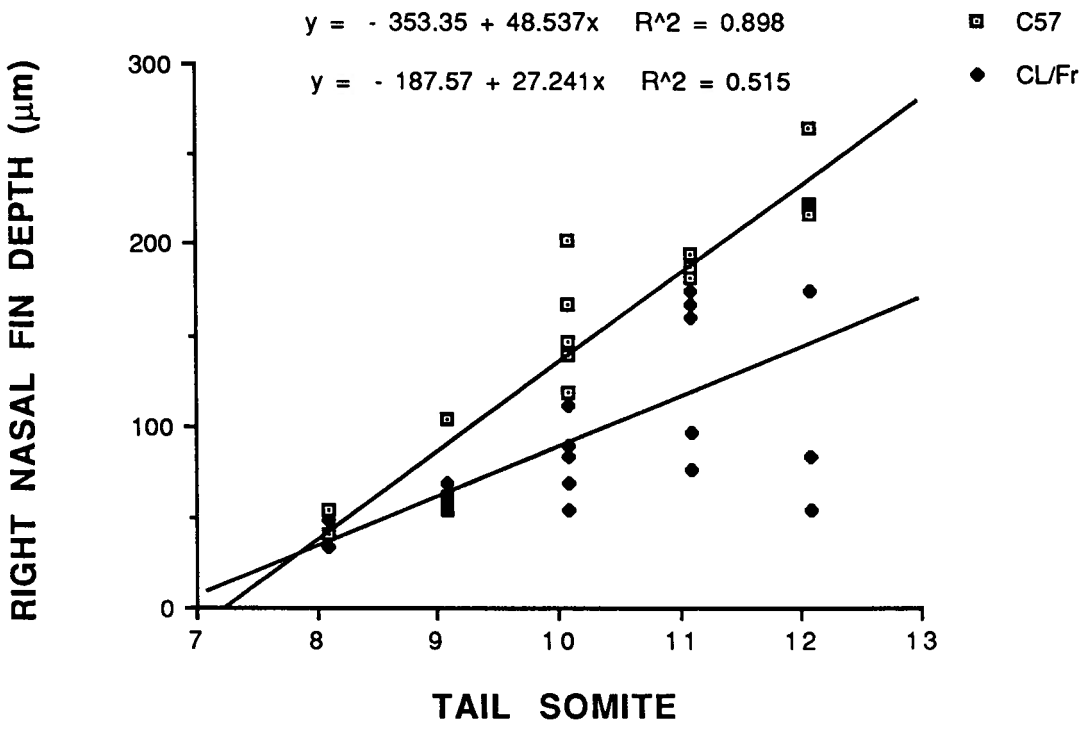


Fig. 12. Regression lines of the right nasal fin depth of C57BL/6J and CL/Fr from 8 to 12 tail somite stage. The growth is slower in CL/Fr than in C57BL/6J ($p < 0.01$) (Table 5). Equations include slopes, intercepts, and squares of correlation coefficients. Linear regressions are not significant in test for lack of fit of C57BL/6J ($F = 2.0$, $p > 0.05$) and CL/Fr ($F = 0.38$, $p > 0.05$).

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: RIGHT NASAL FIN DEPTH

Parameter	Estimate of slope	T -value for hypothesis parameter=0	Probablity for T-value	Standard error of estimate of slope
TS:C57	48.54	7.90	0.0001	6.14
TS:CL/Fr	27.24	4.81	0.0001	5.69
TS*ST C57	21.30	2.53	0.015	0.37
CL/Fr	0.00	.	.	.

Table 5. Test for homogeneity of slopes of right nasal fin from 8 to 12 tail somite stage (TS) between C57BL/6J (C57) and CL/Fr. Regression coefficients of C57BL/6J and CL/Fr are tested for heterogeneity. There is significant difference in the nasal fin depth / tail somite relationship for different strains (ST) by setting to zero effect of the CL/Fr ($p = 0.015$).

maxillary prominence shows that slopes of these two strains are homogeneous and intercepts of these two strains are significantly different (Fig. 13, Table 6).

II. Primary palate development with mesenchymal formation

Five strains were included for studying the regression of nasal fin and mesenchyme formation. In noncleft lip strains BALB/cByJ and C57BL/6J at 13 tail somites, most of nasal fin, which maintains the continuity between the nasal cavity and the roof of the oral cavity, becomes interrupted by the active proliferation of the mesenchyme of the maxillary prominence, lateral nasal prominence and medial nasal prominence, growing across from one side to the other (Table 7) (Fig. 14). This forms the primary palate. In contrast, most of the embryos of the cleft lip strains still maintain the nasal fin at 13 tail somites. The ingrowth of mesenchyme across the nasal fin does not take place until 14 tail somites in A/J, 15 tail somites in A/WySn and 16 tail somites in CL/Fr (Table 7). A CL/Fr embryo of 15 tail somites as shown in Figure 15 without any mesenchyme across the nasal fin may develop into a cleft lip foetus. The internal structures including areas of primary palate and mesenchymal component and the position of maxillary prominence of the five strains of mice of 13-16 tail somites are listed in Appendix 2. The correlation between right and left sides are high in both noncleft lip strains (Table 8) and cleft lip strains except for the mesenchymal component of CL/Fr (0.38) (Table 9). Only 7 of 37 embryos have bilateral mesenchymal formation, 23 embryos have no mesenchymal formation and 7 embryos have mesenchymal formation in one side in CL/Fr. This correlation

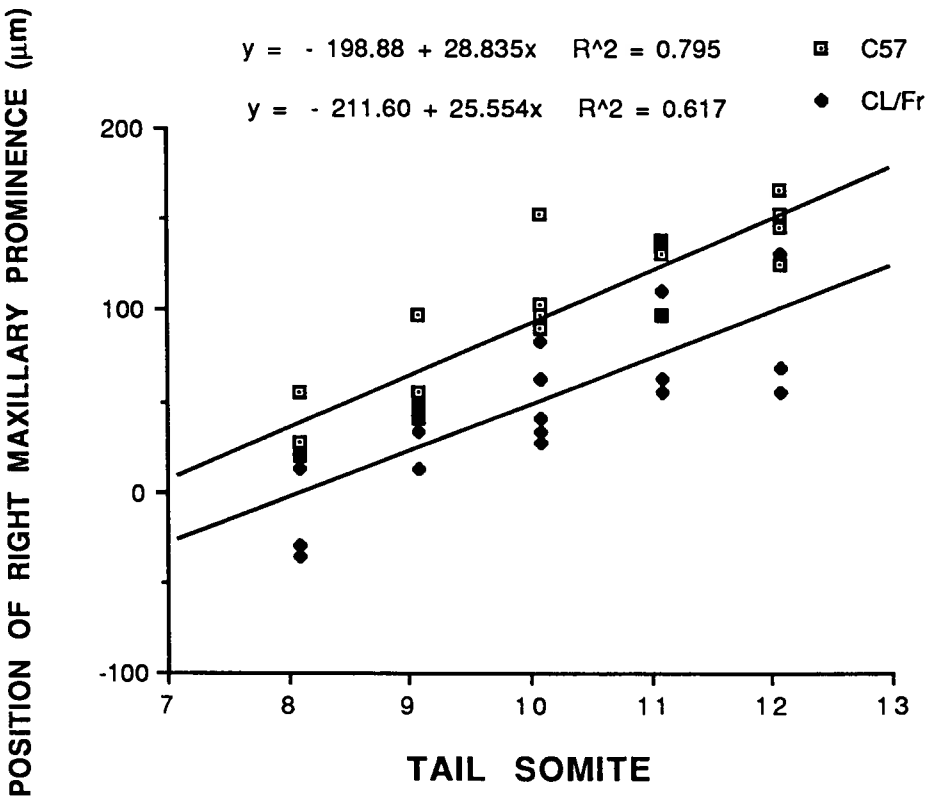


Fig. 13. Regression lines of the position of the right maxillary prominence of C57BL/6J and CL/Fr from 8 to 12 tail somite stage. The growth rates are not significantly different between C57BL/6J and CL/Fr ($p > 0.05$) and the group mean of C57BL/6J is significantly larger than CL/Fr ($p < 0.01$) (Table 6). Equations include slopes, intercepts and squares of correlation coefficients. Linear regressions are not significant in testing for lack of fit of C57BL/6J ($F = 0.78$, $p > 0.05$) and CL/Fr ($F = 1.82$, $p > 0.05$).

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: POSITION OF THE RIGHT MAXILLARY PROMINENCE (RMXP)

LEAST SQUARE MEANS (LSMEAN)

ST	RMXP (μm) LSMEAN	Standard error of LSMEAN	Probability of hypothesis LSMEAN=0	Probability of hypothesis LSMEAN C57= LSMEAN CL/Fr
C57	90.97	5.71	0.0001	0.0001
CL/Fr	45.23	5.21	0.0001	

Parameter	Estimate of slope	T -value for hypothesis parameter=0	Probablity for T-value	Standard error of estimate of slope
TS:C57	28.84	6.96	0.0001	4.14
TS:CL/Fr	25.55	6.66	0.0001	3.84
TS*ST C57	3.29	0.58	0.564	5.65
CL/Fr	0.00	.	.	.

Table 6. Analysis of covariance of the position of the right maxillary prominence of C57BL/6J (C57) and CL/Fr from 8 to 12 tail somite stage (TS). The difference of group least square means between these two strains (ST) is significant ($p < 0.01$). Regression coefficients of C57BL/6J and CL/Fr are tested for heterogeneity of slopes. There is no significant difference in the position of the maxillary prominence / tail somite relationship for different strains by setting to zero effect of the CL/Fr ($p > 0.05$).

	BALB/cBy		C57BL/6J		A/J		A/WySn		CL/Fr	
TS	No. of embryos	% mesenchymal formation	No. of embryos	% mesenchymal formation	No. of embryos	% mesenchymal formation	No. of embryos	% mesenchymal formation	No. of embryos	% mesenchymal formation
13	4	75	5	100	4	0	5	0	7	14
14	7	100	7	100	7	71	6	17	10	10
15	7	100	7	100	8	100	6	50	5	0
16	4	100	7	100	6	83	5	80	15	66

Table 7. The percentage of embryos which have mesenchyme replace the nasal fin of the right side at each tail somite stage (TS) of the five strains.

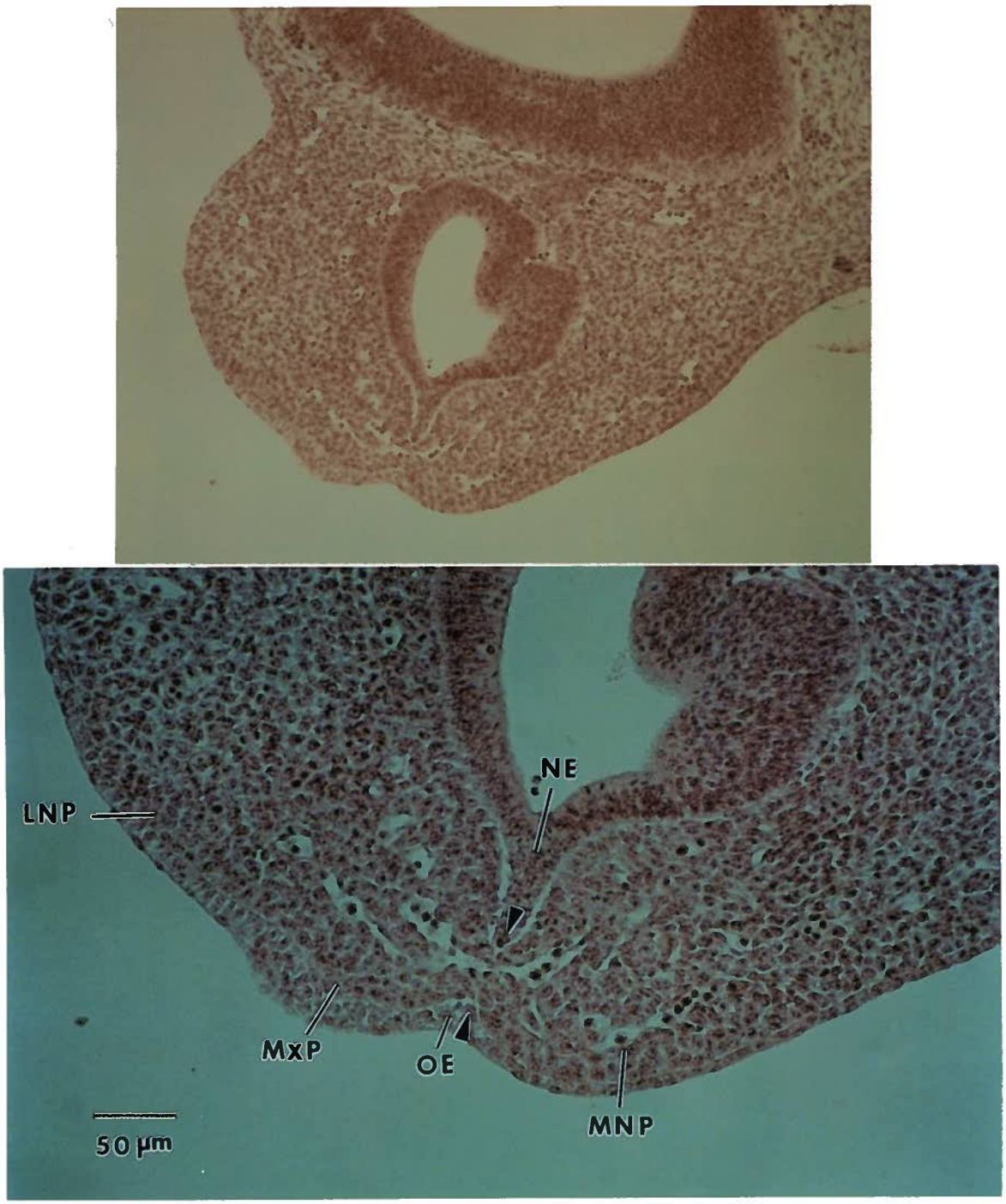


Fig. 14. Frontal section of C57BL/6J showing nasal epithelium, mesenchymal component and oral ectoderm, day 11 hour 0, 13 tail somite stage. The mesenchymal tissue with a blood vessel inside the maxillary, lateral and medial nasal prominence starts to grow across the nasal fin. The nasal fin becomes nasal epithelium (NE) on the top, mesenchymal component in the middle (between the arrow heads) and oral ectoderm (OE) on the bottom. The primary palate area is defined as including these three parts.

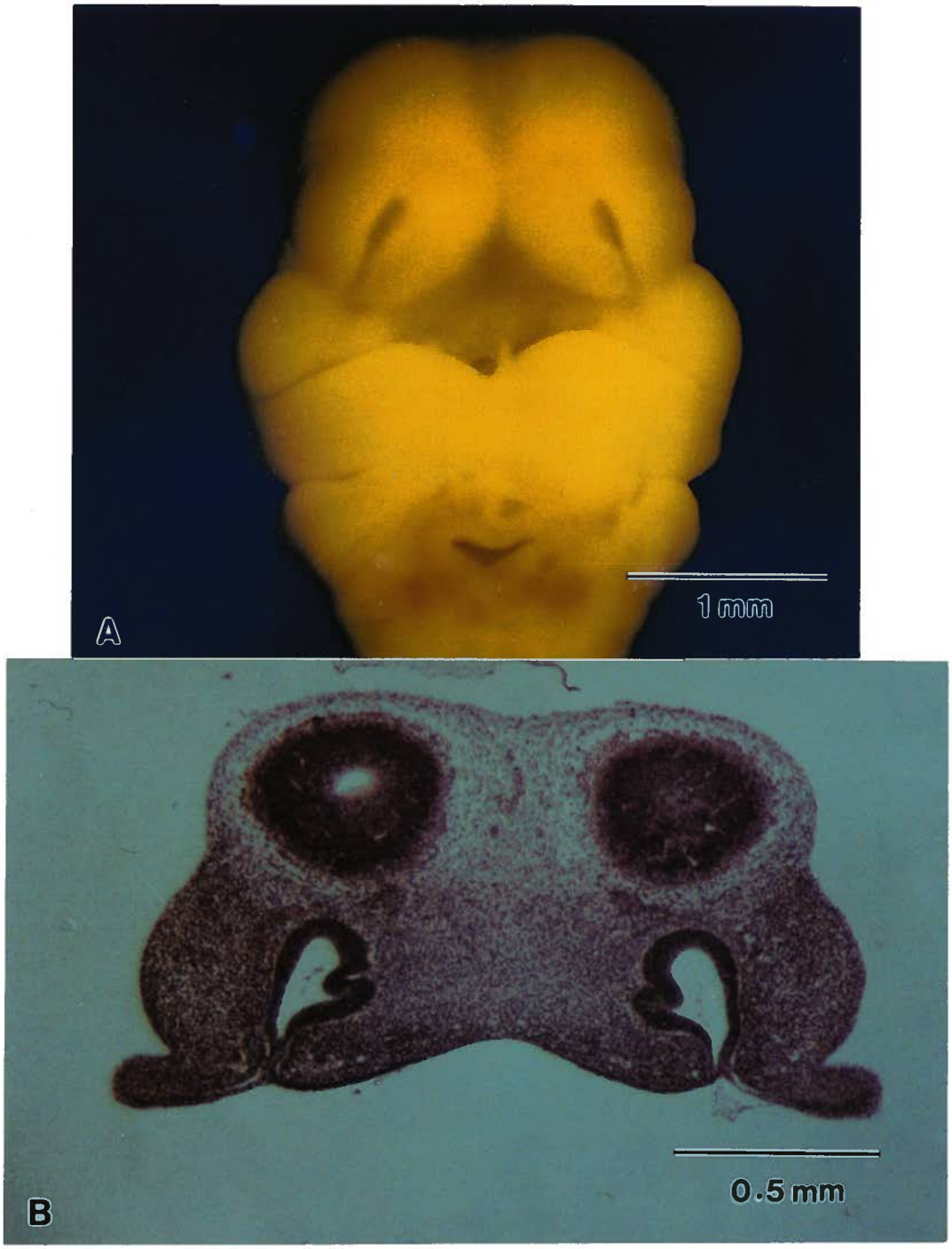


Fig. 15. Frontal view (A) and a frontal section (B) of CL/Fr embryo, day 11 hour 13, 15 tail somite stage. The medial and lateral nasal prominence stay apart from each other (A). The nasal fin is small in the right of the embryo and has barely contact in the left (B). No mesenchymal component formation was found in this section and in this embryo.

PEARSON CORRELATION COEFFICIENTS

BALB/cByJ	N=22					
	LMXP	LPP	LM	LPD	LPH	LMH
RMXP	0.92	0.88	0.83	0.87	0.76	0.84
RPP	0.84	0.92	0.83	0.88	0.81	0.86
RM	0.82	0.88	0.93	0.88	0.73	0.85
RPD	0.84	0.87	0.83	0.92	0.63	0.75
RPH	0.77	0.81	0.69	0.69	0.88	0.85
RMH	0.85	0.90	0.88	0.83	0.87	0.95

C57BL/6J	N=26					
	LMXP	LPP	LM	LPD	LPH	LMH
RMXP	0.93	0.84	0.86	0.64	0.82	0.86
RPP	0.87	0.93	0.90	0.80	0.79	0.87
RM	0.88	0.88	0.93	0.71	0.81	0.89
RPD	0.52	0.70	0.61	0.87	0.35	0.57
RPH	0.82	0.76	0.79	0.43	0.91	0.85
RMH	0.87	0.85	0.89	0.62	0.90	0.93

Table 8. The correlation coefficients between the right and left sides of the maxillary prominence, primary palatal and mesenchymal components in noncleft lip strains from 13 to 16 tail somite stage. The correlation coefficients between the same parameters of the right and left sides are underlined. The abbreviations are explained in Appendix 1.

PEARSON CORRELATION COEFFICIENTS

A/J	N=25					
	LMXP	LPP	LM	LPD	LPH	LMH
RMXP	0.96	0.90	0.77	0.87	0.72	0.84
RPP	0.76	0.90	0.71	0.89	0.80	0.76
RM	0.80	0.75	0.87	0.63	0.62	0.82
RPD	0.69	0.86	0.59	0.94	0.70	0.65
RPH	0.58	0.83	0.60	0.77	0.84	0.64
RMH	0.83	0.83	0.85	0.69	0.71	0.88

A/WySn	N=22					
	LMXP	LPP	LM	LPD	LPH	LMH
RMXP	0.95	0.91	0.69	0.76	0.86	0.68
RPP	0.94	0.92	0.64	0.85	0.89	0.65
RM	0.66	0.64	0.85	0.48	0.65	0.81
RPD	0.78	0.78	0.35	0.88	0.68	0.36
RPH	0.90	0.82	0.65	0.68	0.87	0.67
RMH	0.72	0.75	0.81	0.61	0.71	0.88

CL/Fr	N=37					
	LMXP	LPP	LM	LPD	LPH	LMH
RMXP	0.85	0.77	0.43	0.73	0.76	0.58
RPP	0.81	0.81	0.34	0.79	0.73	0.44
RM	0.59	0.51	*0.38	0.44	0.48	0.54
RPD	0.67	0.68	0.21	0.82	0.55	0.26
RPH	0.84	0.76	0.41	0.66	0.79	0.56
RMH	0.73	0.67	0.56	0.56	0.64	*0.65

Table 9. The correlation coefficients between the right and left sides of the maxillary prominence, primary palatal and mesenchymal components in cleft lip strains from 13 to 16 tail somite stage. The correlation coefficients between the same parameters of the right and left sides are underlined. The abbreviations are explained in Appendix 1.

* Only 7 of 37 embryos have bilateral mesenchymal formation (Appendix 2).

coefficient increases from 0.38 to 0.87 by studying the growth of the mesenchymal component of CL/Fr from 13 to 19 tail somites. Thus, analysis of covariance in the growth of primary palate, mesenchymal component and position of the maxillary prominence of right side only are presented in the data analysis. The increases of the mesenchymal components of the cleft lip strains were studied from 13 to 17 tail somites in A/J and from 13 to 19 tail somites in A/WySn and CL/Fr.

a. Analysis of primary palate area formation

The growth of the primary palate area including both the area replaced and the area not replaced by mesenchyme relative to the tail somite was analyzed among the five strains by the analysis of covariance. The purpose of using covariance analysis is to analyze and compare the growth pattern of the primary palate of the noncleft lip and cleft lip strains. The analysis is to determine if the increases of primary palate area from 13 to 16 tail somites of the five strains have a constant growth rate and if the group least square means of the primary palate area are significantly different among these five strains.

The results of the analysis of covariance shows that slopes of the five strains are homogeneous ($p > 0.01$) and group mean of the primary palate area of BALB/cByJ is significantly larger than those of the three cleft lip strains ($p < 0.01$). In the noncleft lip strains, the group mean of primary palate area of BALB/cByJ is larger than that of C57BL/6J. In the cleft lip strains, A/J has a larger group mean of primary palate area than both A/WySn and CL/Fr while the mean

of A/WySn is also larger than CL/Fr (Fig. 16, Table 10). Linear regressions were also examined for validity in each strain by conducting tests of lack of fit of the linear regression model. All five strains are not significant in these tests ($p > 0.01$). Thus, cleft lip strains have smaller primary palate area than noncleft lip strains during the 13 -16 tail somite stage of development. Each strain in noncleft lip and in cleft lip groups has a significantly larger or smaller primary palate than other strains during the 13-16 tail somite stage of development as well.

b. Partial least squares and covariance analysis of the primary palate area formation

The latent variables of development including tail somite number, body weight, and position of the maxillary prominence were first normalized with 0 as the mean and 1 as standard deviation. Normalized indicators were then multiplied by each simple covariances generated from partial least squares called 'salience' as shown in Table 11. The position of the maxillary prominence has been shown the best predictor for primary palate formation among the three developmental scores (Table 11). Then the summation of each multiplied value was operated to generate the latent variable of development for each embryo. The plot of primary palate area on latent variable of development in each strain shows that this grouping simplifies the linearization of smoothed scatters between primary palate area and latent variable with a higher square of correlation coefficient in each strain (Fig. 17) compared to linear regression between primary palate area and tail somite (Fig. 16), especially in the cleft lip strains. Results of

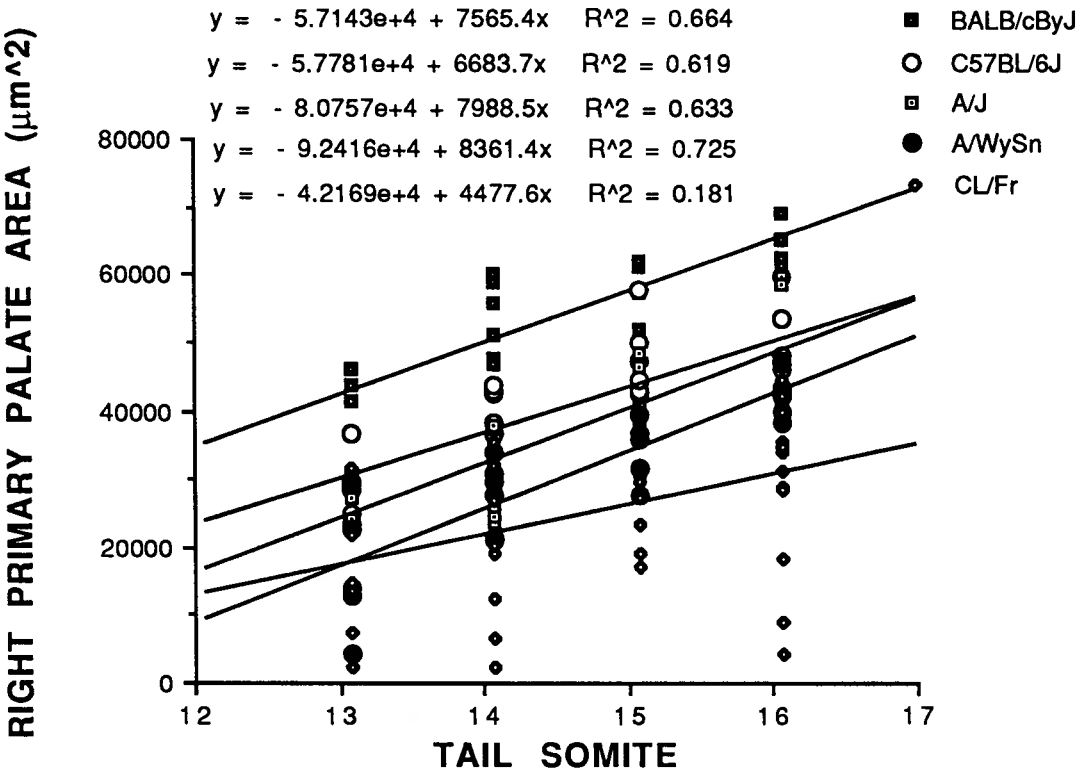


Fig. 16. Regression lines of the right primary palate area of the five strains from 13 to 16 tail somite stage. The keys are in same order as lines. The slopes of five strains are all not significantly different ($p > 0.01$) and the group means of five strains are all significantly different ($p < 0.01$) except between C57BL/6J and A/J (Table 10). Linear regressions are not significant in test for lack of fit of each strain. Equations include slopes, intercepts and squares of correlation coefficients.

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: RIGHT PRIMARY PALATE AREA (RPP)

LEAST SQUARE MEANS (LSMEAN)

ST	RPP (μm^2) LSMEAN	Standard error for LSMEAN	probability of hypothesis LSMEAN=0	Probability of hypothesis:LSMEAN(I)=LSMEAN(J) I/J	1	2	3	4	5
BALB	53250	1705	0.0001	1	.				
C57	39842	1567	0.0001	2	0.0001	.			
A/J	35891	1631	0.0001	3	0.0001	0.0832	.		
A/W	29518	1705	0.0001	4	0.0001	0.0001	0.0079	.	
CL/Fr	22959	1299	0.0001	5	0.0001	0.0001	0.0001	0.0027	.

PARAMETER		Estimate of slope	Test for hypothesis Parameter=0	Probability for T-value	Standard error of estimate of slope
TS*ST	BALB	3087.86	1.52	0.1321	2036.87
	C57	2206.09	1.21	0.2276	1819.19
	A/J	3510.96	1.79	0.0765	1965.32
	A/W	3883.77	2.02	0.0454	1920.95
	CL/Fr	0.00	.	.	.

Table 10. Analysis of covariance of growth of right primary palate area from 13 to 16 tail somite stage (TS) of the five strains (ST). The group least square means are all significantly different among five strains except between A/J and C57BL/6J ($p = 0.0832$). Slopes are not significantly different by setting to zero effect of the CL/Fr ($p \geq 0.0454$).

Latent Variables	BALB	C57	A/J	A/W	CL/Fr
Development					
Tail somite	0.39	0.37	0.38	0.38	0.37
Position of the right maxillary prominence	0.39	0.39	0.58	0.40	0.65
Body weight	0.32	0.34	0.20	0.31	0.18
Primary palate					
Right primary palate area	0.21	0.24	0.22	0.27	0.26
Right mesenchymal area	0.23	0.24	0.25	0.16	0.16
Right primary palate depth	0.21	0.16	0.19	0.25	0.23
Right maximal primary palate height	0.19	0.22	0.17	0.25	0.28
Right maximal mesenchymal height	0.23	0.24	0.27	0.19	0.21

Table 11. Two-block partial least squares analysis of the three developmental scores and the five primary palate parameters blocks of the five strains. The values indicate the simple covariance, saliences, of development and primary palate. The salience is the contribution of each indicator in proportion to its precision. The position of the right maxillary prominence is the more precise estimate and gives more weight in forming the latent variable of development than tail somite and body weight especially in the cleft lip strains.

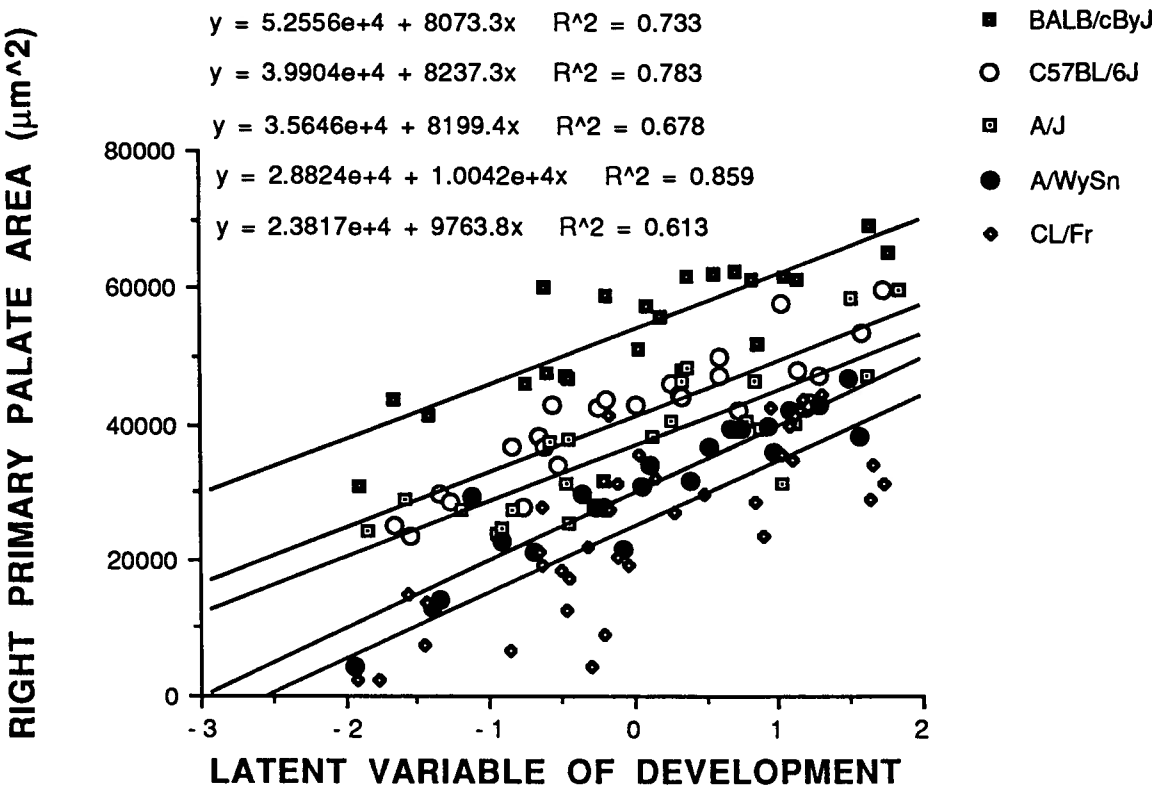


Fig. 17. Regression lines of the right primary palate area on the latent variable of development including the tail somite, body weight and position of the right maxillary prominence of the five strains. The keys are in same order as lines. The slopes of five strains are all not significantly different ($p > 0.01$) and the group means are all significantly different ($p < 0.01$) (Table 12). Regression lines are not significant in test for lack of fit of each strain ($p > 0.05$). Equations include slopes, intercepts, and squares of correlation coefficients. The square of correlation coefficient of CL/Fr increases from 0.181 to 0.613 compared with the regression of right primary palate area on tail somite as shown in Fig. 16 because the right maxillary prominence provides more weight on the growth of primary palate in CL/Fr.

the regression of this area on latent variable of tail somite, body weight, and maxillary prominence also indicate that there is no difference in slope. Also, there are differences by strains in the intercepts of these regressions with the order of BALB/cByJ > C57BL/6J > A/J > A/WySn > CL/Fr ($p < 0.05$) (Table 12). Thus, the delayed formation of the primary palate area can be partly attributed to the delayed forward growth of the maxillary prominence.

c. Analysis of mesenchymal component formation

As shown in Table 7, mesenchymal component formation of BALB/cByJ and C57BL/6J starts at 13 tail somites; 71% A/J at 14 tail somites, 80% A/WySn at 16 tail somites and 67% CL/Fr at 16 tail somites. To verify the analysis of covariance, these five strains have been divided into three groups. The first group (noncleft lip group) is composed of C57BL/6J and BALB/cByJ as most of the embryos at 13 tail somites had mesenchymal formation in both strains. The second group (low cleft lip frequency group) is A/J which shows mesenchymal component formation at 14 tail somites. The third group (high cleft lip frequency group) is composed of A/WySn and CL/Fr as most of the embryos of these two strains have mesenchymal component formation at 16 tail somites. To analyze the formation of the mesenchyme, the noncleft lip and A/J strains were investigated from 13 to 17 tail somites while both A/WySn and CL/Fr strains were investigated from 13 to 19 tail somite animals to generate regression for increases of the mesenchyme. The embryos without mesenchymal component formation were taken away from the data to avoid bias in covariance analysis.

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: RIGHT PRIMARY PALATE AREA (RPP)

LEAST SQUARE MEANS

ST	RPP (μm^2) LSMEAN	Standard error for LSMEAN	probability of hypothesis LSMEAN=0	Probability of hypothesis:LSMEAN(I)=LSMEAN(J)				
				I/J	1	2	3	4	5
BALB	52995	1236	0.0001	1	.				
C57	39922	1137	0.0001	2	0.0001	.			
A/J	35656	1184	0.0001	3	0.0001	0.0105	.		
A/W	28842	1236	0.0001	4	0.0001	0.0001	0.0001	.	
CL/Fr	23835	940	0.0001	5	0.0001	0.0001	0.0001	0.0016	.

PARAMETER		Estimate of slope	Test for hypothesis Parameter=0	Probability for T-value	Standard error of estimate of slope
LVDEV*ST	BALB	-1809.61	-1.15	0.2517	1571.13
	C57	-1526.46	-1.01	0.3140	1509.83
	A/J	-1564.45	-1.03	0.3073	1526.15
	A/W	278.11	0.17	0.8620	1596.51
	CL/Fr	0.00	.	.	.

Table 12. Analysis of covariance of growth of right primary palate area on latent variable of development (LVDEV) of five strains (ST). The group least square means of five strains are all significantly different and regression coefficients are not significantly different by setting to zero effect of the CL/Fr.

Analysis of covariance of two noncleft strains shows homogeneous slope and same intercepts of mesenchyme between these two normal strains ($p > 0.05$). In the high cleft lip frequency group, the same analysis of covariance of growth trends of A/WySn and CL/Fr shows the same slope and same intercepts ($p > 0.05$) between these two high cleft lip frequency strains as well. Between noncleft lip and cleft lip strains, the slopes are homogeneous and intercepts of noncleft lip strains are significantly larger than cleft lip strains (Fig. 18, Table 13). Mesenchymal area of A/J has significantly smaller intercept compared with both BALB/cByJ and C57BL/6J to a common slope and significantly larger intercept than CL/Fr to a common slope (Fig. 18, Table 13). Tests of lack of fit of five linear regressions show that all five strains are not significant ($p > 0.01$).

The mesenchymal areas of these strains were also subjected to analysis of covariance with latent variable of tail somite, body weight and position of the maxillary prominence as covariable. As mesenchymal area data of A/WySn and CL/Fr has been extended to 19 tail somites while the other three strains have been extended to 17 tail somites only, the latent variables are generated for all the strains pooled. Results show that in the five strains, the slopes are homogeneous. The squares of correlation coefficients for regression lines are better than those for regression lines against tail somites only. Within two noncleft lip strains there is no difference in group mean of mesenchymal area between BALB/cByJ and C57BL/6J; in the high cleft lip frequency group, there is no difference in group mean between A/WySn and CL/Fr as well (Fig. 19, Table 14). There is a difference in group means of mesenchymal area between noncleft lip

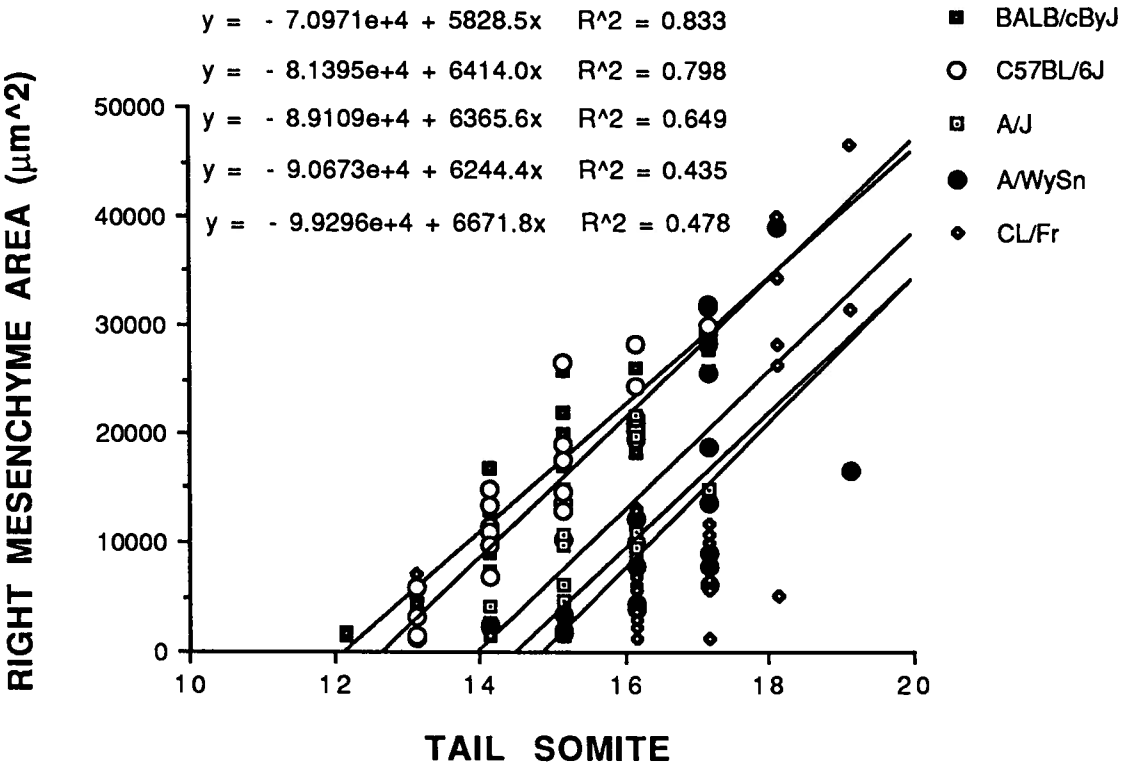


Fig. 18. Regression lines of growth of the right mesenchymal area of the five strains from 12 to 19 tail somite stage. The keys are in same order as lines. They are all not significantly different in slopes. The group means fall into three significantly different groups which are BALB/cByJ and C57BL/6J (noncleft lip strains), A/J (low cleft lip frequency strain) and A/WySn and CL/Fr (high cleft lip frequency strains) (Table 13). The data of 'zero' mesenchyme has been taken out from the analysis to reduce the bias of regression. Linear regressions are not significant in test for lack of fit of each strain ($p > 0.05$) although the fit of A/WySn and CL/Fr to linear regression is poor. Reasons are that very few embryos in the early tail somite stages which have mesenchymal formation in A/WySn and CL/Fr are included in the analysis.

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: RIGHT MESENCHYMAL AREA (RM)

LEAST SQUARE MEANS (LSMEAN)

ST	RM (μm^2) LSMEAN	Standard error for LSMEAN	probability of hypothesis LSMEAN=0	Probability of hypothesis:LSMEAN(I)=LSMEAN(J) I/J	1	2	3	4	5
BALB	19822	1366	0.0001	1	.				
C57	17962	1208	0.0001	2	0.2916	.			
A/J	9540	1449	0.0001	3	0.0001	0.0001	.		
A/W	6054	1586	0.0002	4	0.0001	0.0001	0.1123	.	
CL/Fr	4546	1408	0.0016	5	0.0001	0.0001	0.0170	0.4519	.

PARAMETER		Estimate of slope	Test for hypothesis Parameter=0	Probability for T-value	Standard error of estimate of slope
TS*ST	BALB	-843.35	-0.61	0.5440	1385.36
	C57	-257.85	-0.19	0.8483	1344.56
	A/J	-306.25	-0.17	0.8692	1856.03
	A/W	-427.45	-0.26	0.7954	1644.46
	CL/Fr	0.00	.	.	.

Table 13. Analysis of covariance of growth of right mesenchymal area on tail somite stage (TS) of five strains (ST). The group least square means are significantly different between noncleft lip and cleft lip strains and between A/J and CL/Fr (low and high cleft lip frequency strains). Regression coefficients are not significantly different by setting to zero effect of the CL/Fr.

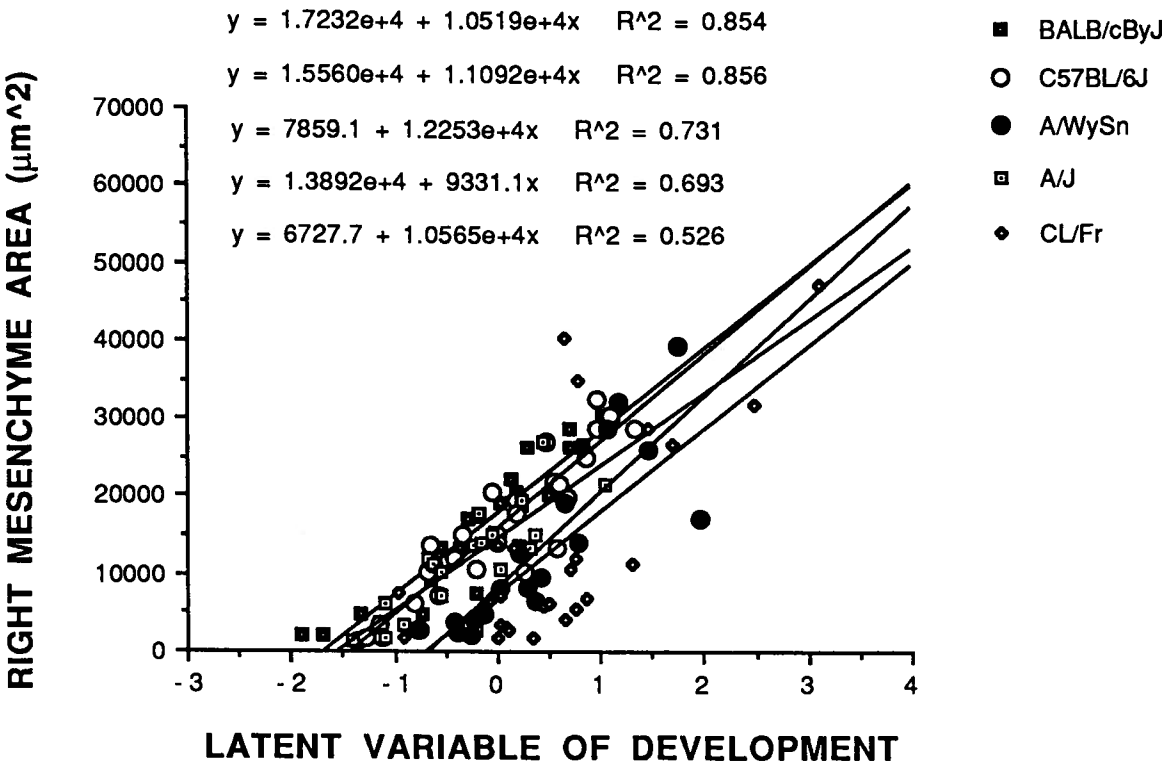


Fig. 19. Regression lines of right mesenchymal area on latent variable including the tail somite, body weight and position of the right maxillary prominence of the five strains. The keys are in same order as lines. The slopes of five strains are homogeneous.The group means also fall into three significantly different groups which are BALB/cByJ and C57BL/6J (noncleft lip strains). A/J (low cleft lip frequency strain) and A/WySn and CL/Fr (high cleft lip frequency strains) (Table 14). The squares of correlation coefficient (R^2) for regression lines are better than those for regression against somites. Linear regressions are not significant in test for lack of fit of each strain. Equations include slopes, intercepts and squares of correlation coefficients.

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: RIGHT MESENCHYMAL AREA (RM)

LEAST SQUARE MEANS (LSMEAN)

ST	RM (μm^2) LSMEAN	Standard error for LSMEAN	probability of hypothesis LSMEAN=0	Probability of hypothesis:LSMEAN(I)=LSMEAN(J) I/J	1	2	3	4	5
BALB	17248	1147	0.0001	1	.				
C57	15437	1031	0.0001	2	0.2403	.			
A/J	12198	1292	0.0001	3	0.0035	0.0511	.		
A/W	8220	1350	0.0001	4	0.0001	0.0001	0.0389	.	
CL/Fr	6465	1178	0.0001	5	0.0001	0.0001	0.0019	0.3172	.

PARAMETER		Estimate of slope	Test for hypothesis Parameter=0	Probability for T-value	Standard error of estimate of slope
LVDEV*ST	BALB	- 45.49	- 0.02	0.9819	1998.68
	C57	527.03	0.28	0.7778	1862.91
	A/J	- 539.33	- 0.22	0.8249	2431.77
	A/W	1688.47	0.78	0.4383	2170.27
	CL/Fr	0.00	.	.	.

Table 14. Analysis of covariance of growth of right mesenchymal area on latent variable of development (LVDEV) of five strains (ST). The group least square means fall into three significantly different groups which are BALB/cByJ and C57BL/6J (noncleft lip strains), A/J (low cleft lip frequency strain) and A/WySn and CL/Fr (high cleft lip frequency strains). Regression coefficients are not significantly different by setting to zero effect of the CL/Fr.

and cleft lip strains and between low cleft lip frequency strain, A/J and high cleft lip frequency strains, A/WySn and CL/Fr. In summary, the mesenchymal areas of the five strains fall into three significantly different groups, noncleft lip strains, low cleft lip frequency, and high cleft lip frequency strains.

d. Analysis of growth of the maxillary prominence

The growth of the maxillary prominence is analyzed in two levels. First, the position of the maxillary prominence versus tail somite is compared by analysis of covariance for cleft and noncleft lip strains. Second, growth of primary palate area and mesenchymal area was investigated by using the position of the maxillary prominence as covariable in analysis of covariance. Growth of primary palatal and mesenchymal area at certain positions of the maxillary prominence was compared within noncleft and cleft lip strains.

Analysis of covariance of the position of the maxillary prominence versus tail somite number indicated no significant difference in the slopes among the five strains. There are differences noticed in intercepts between noncleft lip and cleft lip strains (Fig. 20, Table 15). There is no difference between the noncleft strains BALB/cByJ and C57BL/6J at P = 0.05 level and between the cleft lip strains A/WySn and CL/Fr at p = 0.05 level. The amount of jut of maxillary prominence beyond the end of the nasal fin is greater in A/J than A/WySn and CL/Fr and less than in BALB/cByJ and C57BL/6J at P = 0.05 level (Fig. 20, Table 15).

The frequency of cleft lip in A/J is 4.0% while the cleft lip frequency in A/WySn and CL/Fr is 22.5 and 23.6% as shown in the data. The deficiency of the

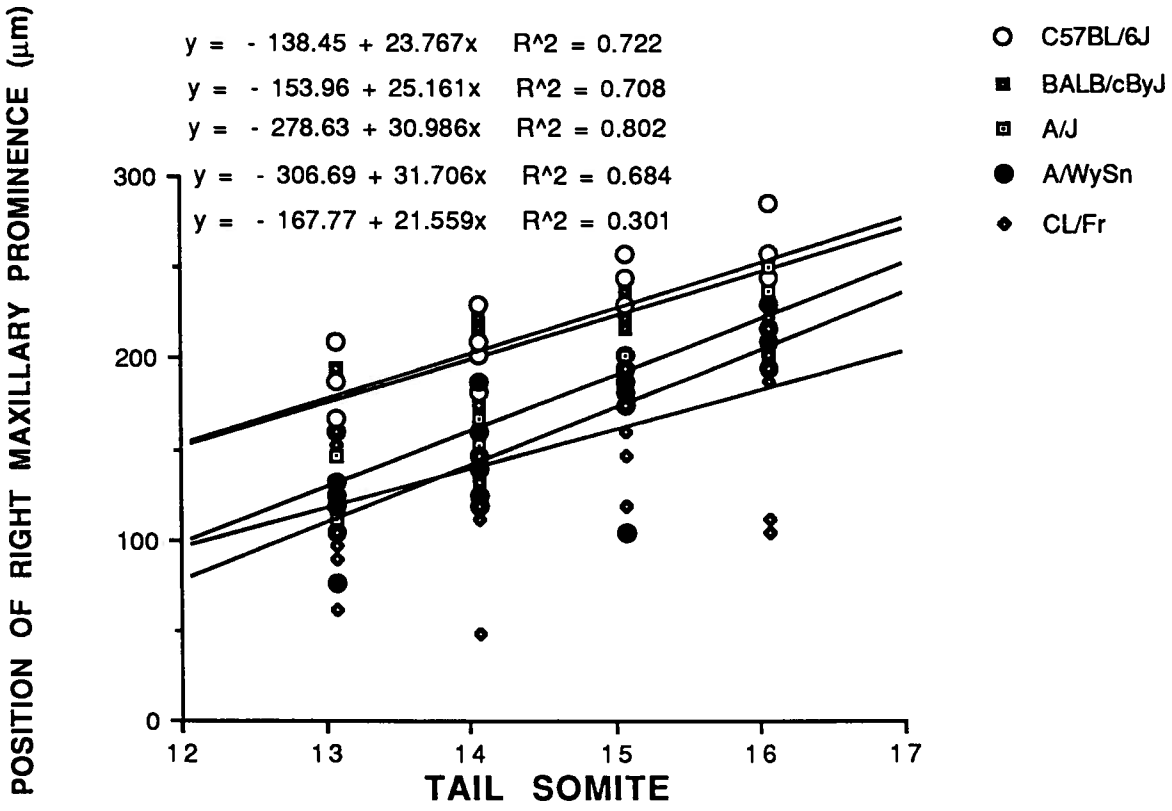


Fig. 20. Regression lines of the position of the right maxillary prominence relative to the end of the nasal fin of the five strains from 13 to 16 tail somite stage. The keys are in same order of lines. The slopes of five strains are parallel and the group means fall into three significantly different groups, noncleft lip strains (BALB/cByJ and C57BL/6J), low cleft lip frequency strain (A/J) and high cleft lip frequency strains (A/WySn and CL/Fr) (Table 15) which are similar to the regressions of mesenchyme area compared with tail somites as shown in Fig. 18. Linear regressions are not significant in test for lack of fit of each strain. Equations include slopes, intercepts and squares of correlation coefficients.

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: POSITION OF RIGHT MAXILLARY PROMINENCE (RMXP)

LEAST SQUARE MEANS (LSMEAN)

ST	RMXP (μm) LSMEAN	Standard error for LSMEAN	probability of hypothesis LSMEAN=0	Probability of hypothesis:LSMEAN(I)=LSMEAN(J) I/J	1	2	3	4	5
BALB	213.41	5.77	0.0001	1	.				
C57	213.52	5.30	0.0001	2	0.9880	.			
A/J	173.84	5.52	0.0001	3	0.0001	0.0001	.		
A/W	155.81	5.77	0.0001	4	0.0001	0.0001	0.0256	.	
CL/Fr	146.53	4.39	0.0001	5	0.0001	0.0001	0.0002	0.2032	.

PARAMETER		Estimate of slope	Test for hypothesis Parameter=0	Probability for T-value	Standard error of estimate of slope
TS*ST	BALB	4.81	0.69	0.4906	6.96
	C57	3.60	0.58	0.5635	6.22
	A/J	9.43	1.40	0.1630	6.72
	A/W	10.15	1.55	0.1248	6.56
	CL/Fr	0.00	.	.	.

Table 15. Analysis of covariance of the position of the right maxillary prominence relative to the end of the nasal fin on tail somite stage (TS) of the five strains (ST). The group least square means are significantly different between noncleft lip and cleft lip strains, between A/J and A/WySn, and between A/J and CL/Fr (low and high cleft lip frequency strains). Regression coefficients are not significantly different by setting to zero effect of the CL/Fr.

maxillary prominence in cleft lip strains can be used to compare with the noncleft lip strains. The deficiency in the high cleft lip frequency group compared with the low cleft lip frequency groups also suggests that the forward growth of the maxillary prominence is significant to the etiology of cleft lip.

The linear regressions of primary palate area on maxillary prominence growth in noncleft and cleft lip strains were compared with analysis of covariance. Results show that regression lines are parallel among five strains. The group mean of noncleft lip (BALB/cByJ) strain is significantly larger than the group means of cleft lip strains, and the group mean of low cleft lip frequency strain (A/J) is larger than those of high cleft lip frequency strains (A/WySn and CL/Fr) (Fig. 21, Table 16). Mesenchymal area is also subjected to the analysis of covariance on maxillary prominence and results show the same differences of group means of mesenchymal areas as those of primary palate areas in five strains (Fig. 22, Table 17).

III. Late primary palate formation and primary choana opening

At 18 tail somites, the oronasal membrane, which is the posterior boundary separating the primary palate from secondary palate, starts to open to form the primary choana in 80% embryos in CL/Fr. However, in C57BL/6J, in only 12.5% of embryos at 18 tail somites does the primary choana start to open. The difference between C57BL/6J and CL/Fr is significant (Chi square test, $p = 0.015$) (Table 18). At 20 tail somites, 80% of embryos in CL/Fr and 70% of embryos in C57BL/6J have the primary choana open. Growth of the nasal fin and subsequent

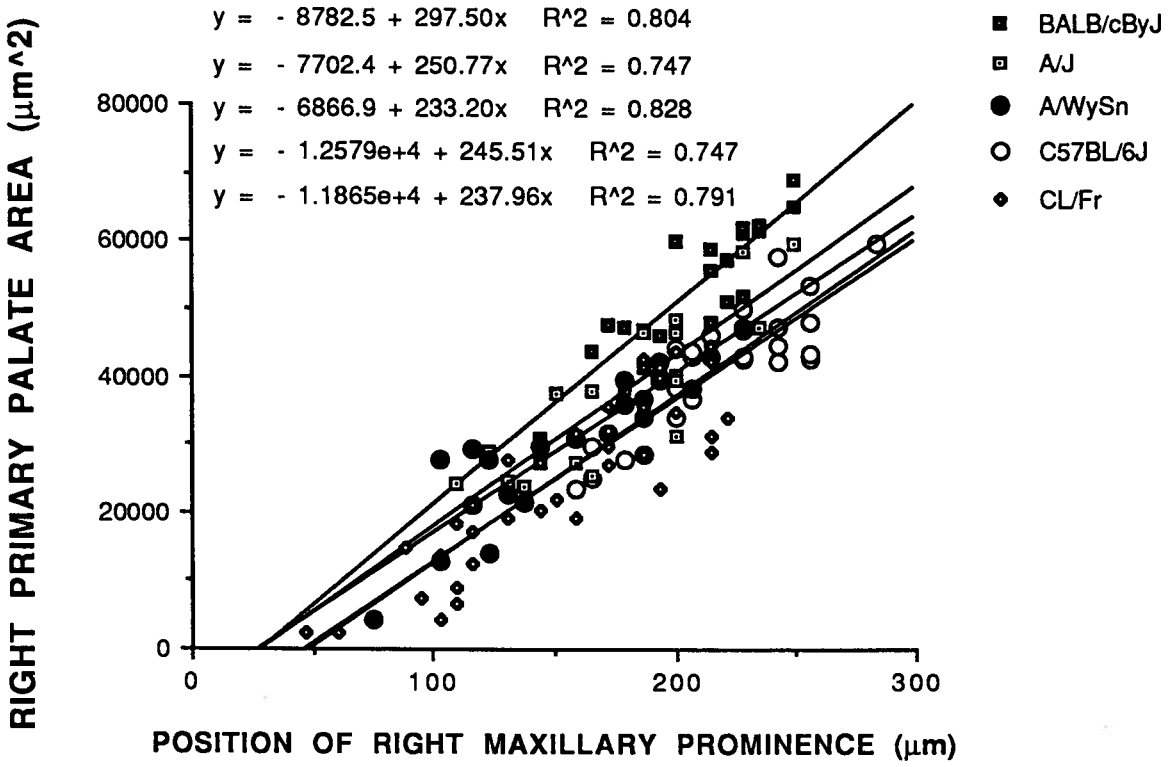


Fig. 21. Regression lines of the right primary palate area on the position of the right maxillary prominence relative to the end of the nasal fin of the five strains. The keys are in same order of lines. The slopes of five strains are homogeneous (Table 16). The group mean of BALB/cByJ is significantly larger than three cleft lip strains and the group mean of A/J (low cleft lip frequency strain) is significantly larger than CL/Fr (high cleft lip frequency strain) (Table 16). Linear regressions are not significant in the test of lack of fit for each strain. Equations include slopes, intercepts and squares of correlation coefficients.

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: RIGHT PRIMARY PALATE AREA (RPP)

LEAST SQUARE MEANS (LSMEAN)

ST	RPP (μm^2) LSMEAN	Standard error for LSMEAN	probability of hypothesis LSMEAN=0	Probability of hypothesis:LSMEAN(I)=LSMEAN(J) I/J	1	2	3	4	5
BALB	44583	1159	0.0001	1	.				
C57	31180	1090	0.0001	2	0.0001	.			
A/J	36734	1047	0.0001	3	0.0001	0.0004	.		
A/W	34659	1129	0.0001	4	0.0001	0.0363	0.1779	.	
CL/Fr	30395	890	0.0001	5	0.0001	0.6015	0.0001	0.0024	.

PARAMETER		Estimate of slope	Test for hypothesis Parameter=0	Probability for T-value	Standard error of estimate of slope
RMXP*ST	BALB	-1.63	-0.04	0.9664	38.59
	C57	7.54	0.21	0.8373	36.69
	A/J	12.81	0.36	0.7222	35.93
	A/W	-4.75	-0.15	0.8843	32.63
	CL/Fr	0.00	.	.	.

Table 16. Analysis of covariance of growth of right primary palate area on the position of the right maxillary prominence (RMXP) relative to the end of the nasal fin of five strains (ST). The group least square means are significantly different between noncleft lip and cleft lip strains, and between A/J and CL/Fr (low and high cleft lip frequency strains). Regression coefficients are not significantly different by setting to zero effect of the CL/Fr.

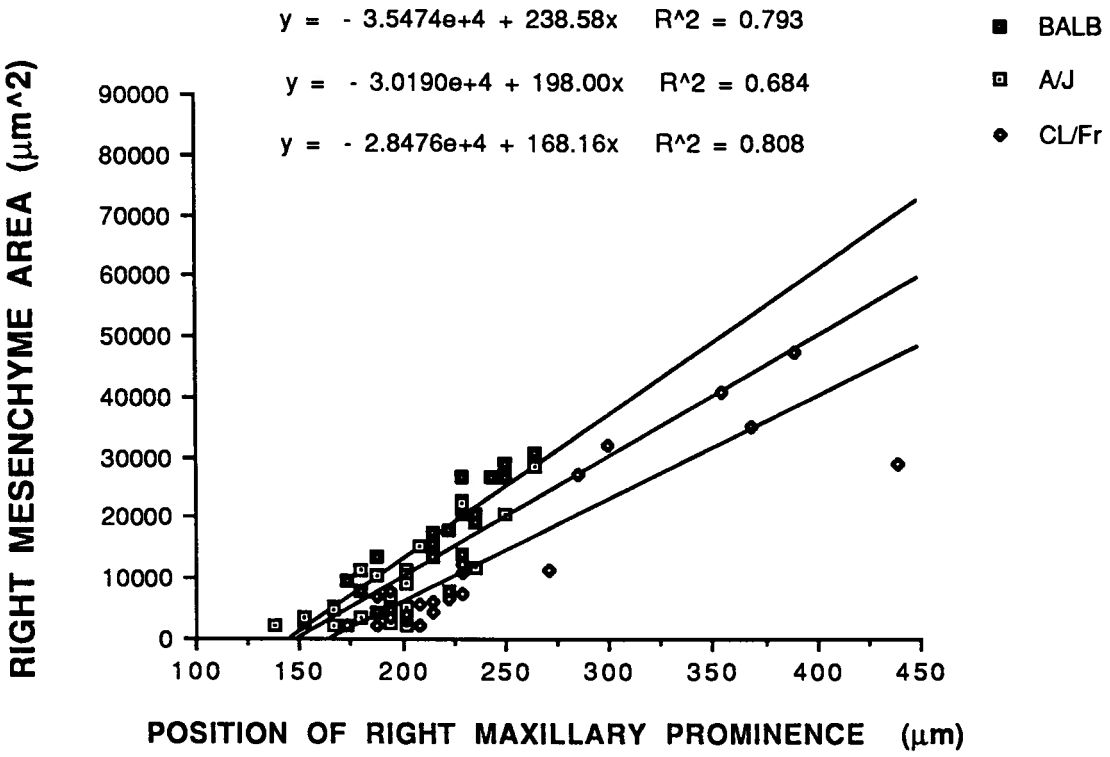


Fig. 22. Regression lines of right mesenchymal area on the position of the right maxillary prominence relative to the end of the nasal fin of the three strains. The keys are in same order of lines. The group mean of BALB/cByJ is significantly larger than cleft lip strains (A/J and CL/Fr) and low cleft lip frequency strain (A/J) is larger than high cleft lip frequency strain (CL/Fr) (Table 17). The slopes are homogeneous among three strains ($p > 0.01$) (Table 17). Linear regressions are not significant in the test of lack of fit for each strain. Equations include slopes, intercepts and squares of correlation coefficients.

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: RIGHT MESENCHYMAL AREA (RM)

LEAST SQUARE MEANS (LSMEAN)

ST	RPP (μm^2) LSMEAN	Standard error for LSMEAN	probability of hypothesis LSMEAN=0
BALB	15945	1046	0.0001
C57	13917	949	0.0001
A/J	12745	1196	0.0001
A/W	10255	1229	0.0001
CL/Fr	7687	1066	0.0001

Probability of hypothesis:LSMEAN(I)=LSMEAN(J)					
I/J	1	2	3	4	5
1	.				
2	0.1546	.			
3	0.0437	0.4458	.		
4	0.0001	0.0200	0.1532	.	
5	0.0001	0.0001	0.0026	0.1142	.

PARAMETER		Estimate of slope	Test for hypothesis Parameter=0	Probability for T-value	Standard error of estimate of slope
RMXP*ST	BALB	71.60	2.02	0.0460	35.47
	C57	66.87	2.15	0.0338	31.10
	A/J	31.03	0.77	0.4414	40.16
	A/W	36.34	1.20	0.2309	30.16
	CL/Fr	0.00	.	.	.

Table 17. Analysis of covariance of growth of right mesenchymal area on the position of the right maxillary prominence (RMXP) relative to the end of the nasal fin of five strains (ST). The least square means are significantly different between noncleft lip and cleft lip strains and between A/J and CL/Fr (low and high cleft lip frequency strains). Regression coefficients are not significantly different by setting to zero effect of the CL/Fr ($p > 0.01$).

	C57BL/6J			CL/Fr		
Tail Somites	No of embryos	No. of primary choanae opened	% of primary choanae opened	No of embryos	No. of primary choanae opened	% of primary choanae opened
17	4	0	0	6	1	17
18	8	1	*12.5	5	4	80
19	8	2	25	2	2	100
20	9	7	78	5	4	80

Table 18. Percentage of embryos with primary choanae opened in embryos of 17-20 tail somites in C57BL/6J and CL/Fr strains.

* The percentage of primary choanae opened in CL/Fr is significantly greater than in C57BL/6J at 18 tail somite stage (Chi square test. $p < 0.05$).

mesenchymal replacement and enlargement are anticipated in both noncleft and cleft lip strains from 13 to 16 tail somites. The opening of the primary choanae indicates a definite primary palate formation for CL/Fr at 18 tail somites and for C57BL/6J at 20 tail somites. The results showed that there is a longer interval for mesenchymal ingrowth and enlargement in C57BL/6J from 13 tail somites to 20 tail somites than in CL/Fr from 13 tail somites to 18 tail somites.

DISCUSSION

A. Cleft lip frequency and resorption rate

I. Strain effect

The present study demonstrates that the frequency of cleft lip falls into two groups; A/J is the low cleft lip frequency (4.0%) group; A/WySn and CL/Fr are in the high cleft lip frequency group (22.5% and 23.6%). The frequency of resorption of the low cleft lip frequency strain (A/J) is higher than high cleft lip frequency group (18.0% of A/J versus 5.3% of A/WySn and 12.2% of CL/Fr). Thus a reciprocal relationship is expressed among these three strains. A/WySn and CL/Fr both have a higher frequency of cleft lip and a lower resorption rate compared with the lower frequency of cleft lip and higher rate of resorption of A/J.

In previous studies of fetal A/J mice, the frequency of cleft lip was found to be 8.5% (Trasler, 1960) and 7.9% (Juriloff, 1982). In studies of fetal A/WySn mice, the frequency of cleft lip was 29% (Juriloff, 1982), and 19.5% (Juriloff and Harris, 1985). Studies of fetal CL/Fr mice showed frequencies of 21.2% (Juriloff and Fraser, 1980), 21% (Juriloff, 1981), and 26% (Staats, 1972). There is good agreement between previous studies and this one for A/WySn and CL/Fr for percent CL(P). The frequency of CL(P) in A/J is known to have dropped since 1980 (Trasler and Trasler, 1984). This difference in frequency of cleft lip among the A strains of mice may be due to a genetic maternal effect (Juriloff, 1982). In the study by Juriloff (1982), the cleft lip frequency in A/J was 9.9% and the resorption rate was 24.8%, while in CL/Fr the cleft lip frequency was 21.2% and the resorption rate was 5.8%. It was suggested that maternal effects account for

the difference and that the maternal trait may be the difference in survival rate of cleft lip fetuses.

Present results show CL(P) frequencies similar to those in previous reports. The results also provide a novel model for comparing the development of palatal structures of higher and lower frequency cleft lip strains as well as comparing normal and cleft lip strains of mice. The hypothesis is that the high frequency of cleft lip in the A/WySn and CL/Fr results from deficient primary palatal structure in these two strains compared with A/J which has low cleft lip frequency. Another hypothesis is that the low cleft lip frequency in A/J results from the higher mortality of embryos which may develop cleft lip. Since these cleft lip A/J embryos may die due to the maternal effect, there are less cleft lip embryos surviving in A/J litters than in A/WySn and CL/Fr litters. These surviving cleft lip embryos in A/WySn and CL/Fr may have more severe deficiencies of the palatal structures than A/J embryos.

II. Uterine site effect

It has been found that within the A/J strain, embryos in the uterine site nearest the ovary develop cleft lip significantly more often than embryos in other positions in the uterine horn (Trasler, 1960). Kalter (1975) found that the frequency of cleft lip was higher at both the ovarian and cervical sites and resorption was lower at the ovarian site. Juriloff (1980) has reinvestigated this uterine site effect and shown increased cleft lip and decreased resorption at the ovarian site. It is suggested that a relatively privileged area at ovarian site in both

A/J and CL/Fr allows the survival of cleft lip embryos that would have died elsewhere (Juriloff, 1980). In this study, cleft lip frequency is significantly higher at the ovarian site than at other sites when three cleft lip strains are analyzed together, but the resorption rate is not significantly different between these two sites. This pattern was present in each of the strains but the sample size limited identification of statistical significant differences. By analyzing three cleft lip strains together my findings support the hypothesis that the implantation site has an effect on frequency of cleft lip but not on resorption rate. The effects of implantation site on the size of primary palate and the mesenchyme will be investigated in the future. The hypothesis is that the embryos at ovarian sites tend to have less developed primary palates than embryos at other uterine sites.

B. Tail somite stage and chronological age

The two cleft lip strains (A/J and A/WySn) belonging to the highly inbred A/- strain and one cleft lip strain (CL/Fr) which is related to the A/- strain are studied. The A/-strain is derived from a cross between Cold Spring Harbor albino and Bagg albino as long as 50 years ago (Staats, 1972; Bailey, 1978). The present investigation demonstrates that at equivalent tail somite stages the chronological ages of these three cleft lip strains are similar to those of BALB/cByJ originating from albino (white coat with pink eyes) breeding stock without spontaneous cleft lip. They are thought to share more alleles with the A stock than C57BL/6J (Taylor, 1972). C57BL/6J is a very distinctive inbred strain on the basis of the average number of shared alleles with the other four strains

(Taylor, 1972). In the present study, the chronological age of C57BL/6J is younger (from D10/17 to D11/8) than those of other four strains (from D11/2 to D11/18) at the same tail somite stage (8 to 18 tail somites). The differences in chronological age distribution can be explained on the basis of different genetic background.

C. Delineation of phases of primary palate development

I. Early primary palate development

The data from 8 to 12 tail somites show that forward growth of the maxillary prominence is retarded in CL/Fr compared with C57BL/6J. At 8 tail somites, the maxillary prominence of C57BL/6J extends frontally past the end of the nasal fin and joins with the lateral nasal prominence to form the lateral wall of nasal pit which fuses with epithelium of medial nasal prominence. In contrast, the maxillary prominence of CL/Fr is still left behind the end of the nasal fin at the same stage. From 9 to 12 tail somites, the maxillary prominence of CL/Fr grows frontally over the end of nasal fin, but the depth is significantly smaller than the depth in C57BL/6J. These results confirm previous studies suggesting that regional growth deficiency or developmental abnormality in the maxillary prominence may be a common feature in primary palatal clefting (Reed, 1933; Johnston and Hunter, 1989, Diewert and Shiota, 1990).

The results of nasal fin depth measurements are similar to the results of Reed (1933) who measured the nasal floor (fused portion) of the nasal fossa in a small number of cleft lip embryos and their normal littermates. His results have

shown a floor length of 90 μm in cleft lip mice and of 320 μm in normal mice of 11 days 2.5 hours. The present study of 11 day old mice has shown the length of nasal fin, which is the fused portion, ranged from 40 to 220 μm in C57BL/6J and from 30 to 120 μm in CL/Fr.

Analysis of covariance in the depth of nasal fin in C57BL/6J and CL/Fr from 8 to 12 tail somites suggests that the growth rate is different ($p < 0.05$) between these two strains and growth of the nasal fin of CL/Fr is slower than that of C57BL/6J. On the other hand, the analysis of covariance on the position of maxillary prominence results in a homogeneous growth rate of two strains with the forward growth of maxillary prominence being delayed in CL/Fr ($p < 0.05$). The slower growth rate of the nasal fin depth in CL/Fr compared to C57BL/6J is probably a result of deficient nasal fin depth in several embryos of CL/Fr which will probably end with complete or partial cleft lip as shown in Figure 11. The growth rate of the nasal fin is slower in CL/Fr compared with C57BL/6J and the size of the maxillary prominence is smaller, however, the growth rate of the maxillary prominence is similar in the two strains (Fig. 12). Thus the delayed forward growth of maxillary prominence in CL/Fr appears to contribute partially to the deficiency of the nasal fin. Also this result partially supports the conclusion from Reed (1933) that the failure of nasal prominences to fuse with each other is attributable to a retarded maxillary prominence growth.

II. Primary palate development with mesenchymal component formation.

a. Primary palate area formation.

From 13 to 16 tail somites, mesenchyme forms at the epithelial nasal fin in these five strains. It forms earlier in noncleft lip strains (13 tail somites) than in cleft lip strains (14 to 16 tail somites). The primary palate area including both epithelial and mesenchymal areas were analyzed to understand the general mechanisms involved during primary palate development. Comparing the increase of right primary palate area to tail somite number in five strains I found the growth rate of primary palate area of five strains to be the same ($p \geq 0.05$). Group means of right primary palate areas of noncleft lip strain (BALB/cByJ) are significantly larger than those of the three cleft lip strains. In the three cleft lip strains, group means of the low cleft lip frequency strain (A/J) are larger than those of high cleft lip frequency strains (A/WySn and CL/Fr) at this critical stage of primary palatogenesis.

Although one major gene has been shown involved in the expression of CL(P) in mice (Juriloff, 1986; Biddle and Fraser, 1986), there are controversies about the effect of the gene. The expression of this major gene could be expressed as developmental deficiencies in different areas of the facial prominences in the cleft lip strains. Both deficient growth of the maxillary prominence and the less divergent medial nasal prominence could lead to deficiency in contact between the lateral and medial nasal prominences (Reed, 1933; Trasler, 1968). These biological traits are possible factors contributing to the smaller primary palatal area in the cleft lip strains than noncleft strains.

In addition, Millicovsky *et al* (1982) and Forbes *et al* (1989) have shown that lack of divergence of medial nasal prominences and the depressed activity of

surface epithelium may contribute to the higher cleft lip frequency observed in CL/Fr and A/WySn than in A/J. A/J embryos show no depressed activity of the surface epithelium. My results showing significantly smaller internal nasal contact area in A/WySn and CL/Fr than in A/J support the idea that A/J with lower cleft lip frequency quantitatively has less failure of contact than A/WySn and CL/Fr with higher cleft lip frequency. This reduced failure of contact in A/J mice compared to A/WySn and CL/Fr may be associated with an additional depressed ability of the surface epithelium in A/WySn and CL/Fr. The maternal effect on the uterus through the serum of the pregnant mice results in more embryos having cleft lip in A/WySn and CL/Fr and fewer embryos having cleft lip in A/J. Since the resorption rate is higher in A/J mice than in A/WySn and CL/Fr mice, the higher frequency of CL(P) in A/WySn and CL/Fr compared with A/J may be explained by the possibility that the resorbed embryos in A/J mice may have gone on to develop cleft lip had they not been resorbed. Depressed activity of the epithelium observed in A/WySn (Forbes *et al.* 1989) and CL/Fr (Millicovsky *et al.*, 1982) embryos compared with A/J embryos may also contribute to the smaller internal contact area in these strains.

Both BALB/cByJ and C57BL/6J are noncleft lip strains; however, the primary palate area is significantly smaller in C57BL/6J than in BALB/cByJ. The genetic background of C57BL/6J is very distinctive on the basis of the average number of shared alleles with other strains including BALB/cByJ (Taylor, 1972). In addition, the chronological age is younger in C57BL/6J than in BALB/cByJ at a similar tail somite number (Fig. 4). Thus, the smaller primary palate area in

C57BL/6J compared to BALB/cByJ may result from the different genetic background between these two inbred strains.

A/WySn has a larger primary palate area than CL/Fr, although these two strains have the same cleft lip frequency. The CL/Fr strain is derived from a heterogeneous stock crossed to A/J and inbred by brother-sister mating with selection for high frequency of spontaneous cleft lip (Bornstein *et al*, 1970). Millicovsky *et al* (1982) reported that after primary fusion fails to consolidate the area at the bottom of the nasal pit at 6 tail somites from the genital tubercle (equivalent to 10 tail somites in this study), CL/Fr embryos have a second opportunity to fuse their primary-palate primordia at 10 tail somites (equivalent to 14 tail somites in this study). As the nasal prominences continue to grow in size, in approximately two-thirds of the embryos, the medial and lateral nasal prominences gain close apposition. This contact may be preceded by isolated bursts of epithelial activity in regions adjacent to the initial fusion area. The authors suggested that the process of this "secondary fusion" may facilitate the successful fusion of the primary palate in CL/Fr embryos without cleft lip. This secondary fusion may only exist in CL/Fr and not in A/WySn. Although the primary palate contact area is larger in A/WySn than in CL/Fr before the mesenchymal component forms, some CL/Fr embryos will catch up later and form a successful primary palate through secondary fusion. Consequently the frequency of cleft lip between CL/Fr and A/WySn is similar. Further study of A/WySn and CL/Fr embryos is needed to test this hypothesis.

b. Primary palate area formation and embryonic development

The principles of partial least square analysis as they apply to morphometric and developmental studies (Bookstein, 1991) operate to optimize the covariance available for effective statistical predictions in complex systems. For instance, developmental index is taken not as a single indicator but as a latent variable (LV) combining the tail somite number, body weight, and position of the maxillary prominence relative to the end of the nasal fin. Because these indicators are correlated quite strongly over samples of any range of maturities, a composite of these scores can be expected to show a stronger pattern of covariance with morphometric outcomes, and thereby to underlie more precise analyses of primary palate area, than that available by reference to any single measure (Bookstein *et al*, 1985).

For the relation between the three developmental indicators and the five primary palate parameters, the developmental LV is dominated by maxillary prominence position variable, especially in the cleft lip strains (with greater weight placed on the developmental measures) (Table 11). The primary palate latent variable is more evenly weighted across all the measures of parameters. The regression coefficient of primary palate area on latent variable in each strain is better than the regression coefficient of primary palate area on tail somite number, especially in the cleft lip strains (compare Fig. 16 and Fig. 17). Thus, this grouping simplifies the linearization of smoothed scatters between LV and the primary palate areas because forward growth of the maxillary prominence is a better predictor for primary palate area formation than tail somite and body

weight, especially in cleft lip strains.

c. Temporal and spatial analysis of mesenchymal replacement of the epithelial seam

The mechanisms by which the mesenchyme replaces the epithelial seam remain poorly understood. The growing-through of the epithelial plate by active proliferation of the mesenchyme has been proposed to follow fusion of medial nasal, lateral nasal and maxillary prominences (Warbrick, 1960; Trasler, 1968; Vermeij-Keers, 1972; Gaare and Langman, 1977b). Different mechanisms proposed for loss of the epithelial seam include programmed peridermal cell death and transformation of basal epithelial cells to mesenchymal cells (Fitchett and Hay, 1989). Anderson and Matthiessen (1967) have suggested that complete cleft lip will appear if mesenchymal proliferation is retarded, and cleft lip with a mesenchymal bridge will appear in cases where there is less marked retardation of the mesenchymal proliferation. The results of this study of mesenchymal replacement of the nasal fin relative to developmental age marked by tail somite stages show that mesenchymal component formation is delayed in cleft lip strains compared with noncleft lip strains. Seventy five percent of embryos of noncleft lip strain (BALB/cByJ) start mesenchymal replacement at 13 tail somites. Cleft lip strains start mesenchymal replacement at 14 tail somites in 70% of the embryos of A/J, at 16 tail somites in 80% of the embryos of A/WySn and in 70% of the embryos of CL/Fr. Thus, mesenchymal replacement is delayed in the cleft lip strains, and the replacement by mesenchyme of low clefting frequency (A/J) strain

was less retarded than the high clefting frequency strains (A/WySn and CL/Fr).

This study also provides a quantitative analysis of mesenchymal component formation. The analysis of covariance of the mesenchymal area from 13 to 17 tail somites in BALB/cByJ and C57BL/6J, from 14 to 17 tail somites in A/J and from 15 to 19 tail somites in A/WySn and CL/Fr includes mesenchymal proliferation and enlargement in the five strains. Results have shown that the slopes of the five strains are homogeneous, suggesting the growth rates of the mesenchymal components of noncleft and cleft lip strains are not different. The significantly smaller mesenchymal area in the cleft lip strains compared to the noncleft lip strains indicates that mesenchymal growth in cleft lip strains is retarded. There is a significantly smaller mesenchymal area in the high cleft lip frequency strains (CL/Fr) compared with the low cleft lip frequency strain (A/J). This suggests that the formation and enlargement of the mesenchymal area is a specific indicator for determining the cleft lip malformation in noncleft lip, low cleft lip frequency and high cleft lip frequency strains of mice.

The partial least squares analysis has also been applied for comparing mesenchymal areas of noncleft and cleft lip strains. Because the tail somite interval is different among the five strains, normalization of indicators for latent variables were based on all the strains to avoid hiding the delayed mesenchymal component formation relative to the tail somite number. The results indicate that the five strains can be divided into three significantly different groups of mesenchymal component formation, noncleft lip strains (BALB/cByJ and C57BL/6J), low cleft lip frequency strain (A/J) and high cleft lip frequency strains

(A/WySn and CL/Fr). Thus the strain effect on the cleft lip frequency is the same as the strain effect on mesenchymal component formation. The results also show that the regression coefficients for mesenchymal area in each strain are better when latent variables, rather than tail somite number, are used as the covariable (compare Figure 18 and Figure 19).

d. Analysis of the growth of maxillary prominence

The relationship of the position of the maxillary prominence relative to the nasal fin, epithelial fusion and mesenchymal replacement is still not well understood. The present results show that the forward growth of the maxillary prominence had the same pattern as mesenchymal replacement in the strains. The maxillary prominence is in a more advanced position in a noncleft lip strain than in cleft lip strains. Comparing the low cleft lip frequency strain (A/J) with high cleft lip frequency strains (A/WySn and CL/Fr), the former is significantly more advanced than the latter two. The less advanced position of the maxillary prominence in the high cleft lip frequency strain is clearly associated with a higher cleft lip frequency. In addition, the maternal effect on the higher survival rate of cleft lip embryos in A/WySn and CL/Fr compared to A/J results in more embryos having cleft lip in A/WySn and CL/Fr and fewer embryos having cleft lip in A/J. Since the resorption rate is higher in A/J mice than in CL/Fr mice, the higher frequency of cleft lip in A/WySn and CL/Fr may be explained by the possibility that the resorbed embryos in A/J mice may have gone on to develop cleft lip had they not been resorbed. Thus, more cleft lip embryos surviving with

less advanced position of the maxillary prominences in high cleft lip frequency strains (A/WySn and CL/Fr) than in the low cleft lip frequency strain (A/J) may explain the less advanced mean position of maxillary prominence in the high cleft lip frequency strains.

A further question that has to be answered is whether a retarded maxillary prominence can explain retarded primary palate formation. The primary palate and mesenchymal area are both analyzed with the position of the maxillary prominence as a covariable for testing the strain effect. Results show that at a certain position of maxillary prominence, the primary palate and mesenchymal areas are still significantly larger in the noncleft lip strain (BALB/cByJ) than in the cleft lip strains. However, when tail somites is used as the covariable in analysis of covariance, the difference between the group means in the noncleft lip strain and the cleft lip strains is only half as large. In other words, at a certain tail somite number the difference between noncleft lip and cleft lip strains can be partially explained by the retarded maxillary prominence. Other possible pathogeneses of these cleft lip strains include: less divergent medial nasal prominences in cleft lip strains than in noncleft lip strains (Trasler,1968; Juriloff and Trasler, 1976), and depressed ability of surface epithelium in high cleft lip strains (A/WySn and CL/Fr) but not in a low cleft lip frequency strain (A/J) (Millicovsky *et al*, 1982; Forbes *et al*, 1989).

In summary, a single recessive gene involved in the expression of CL(P) may be expressed in different developmental deficiencies of facial prominences during primary palate formation. As shown in Figure 23, this single gene may be

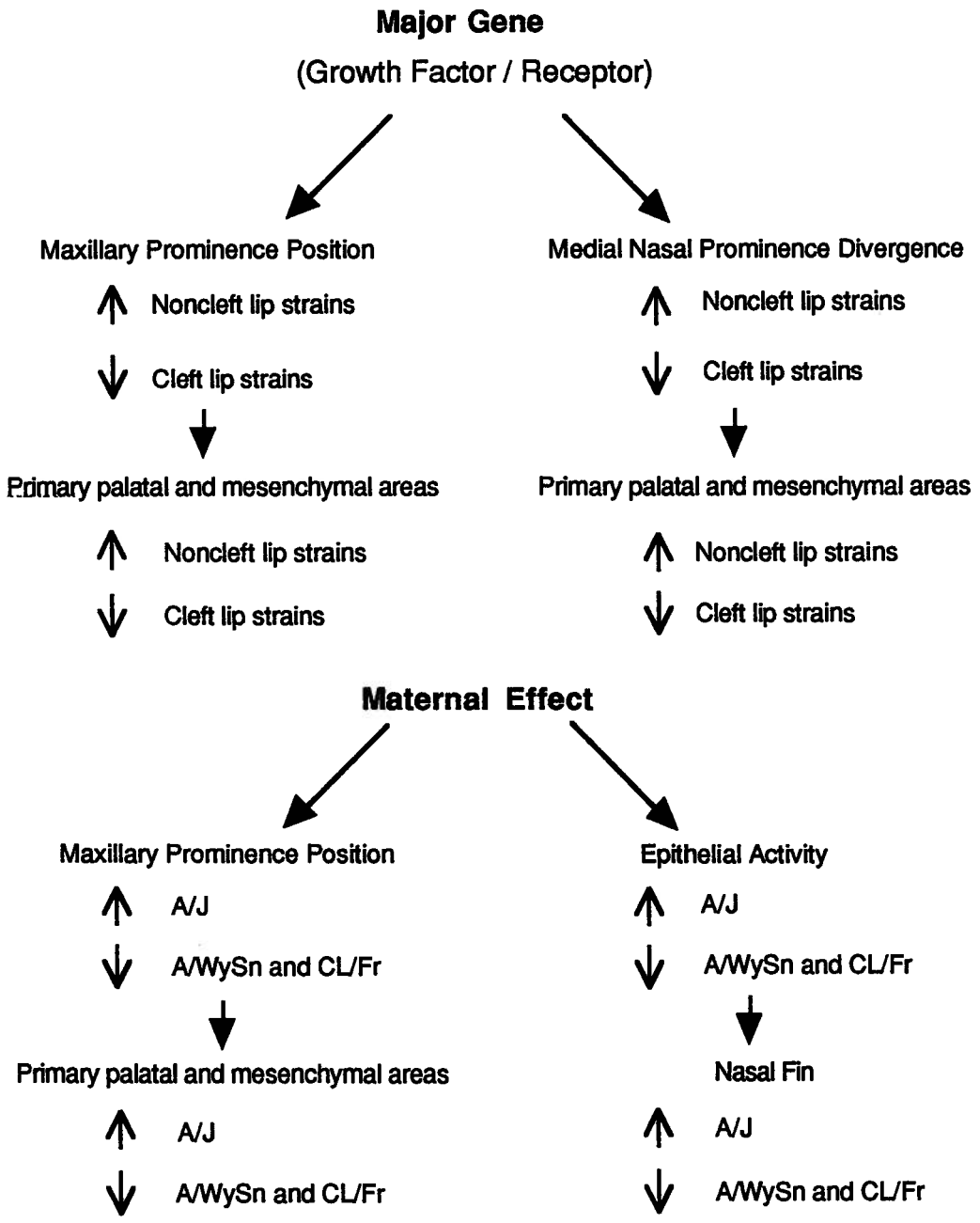


Fig. 23. Summary of hypotheses of cleft lip gene effects and maternal effects.

related to a growth factor or a receptor of a growth factor. The damage of the normal function affecting expression of a growth factor, a receptor or other gene product may lead to: retarded forward growth of the maxillary prominence, less divergent medial nasal prominences or deficient medial nasal prominences. The difference of primary palatal and mesenchymal component area between the noncleft lip and cleft lip strains can be partially explained by the retarded forward growth of the maxillary prominence and partially by the less divergent or deficient medial nasal prominences. Another genetic factor related to the difference of cleft lip frequency in the cleft lip strains is the maternal effect (Fig. 23). In humans, maternal effects have been shown to increase the frequency of CL(P) in children of White mothers of mixed Black/White parentage (Khoury *et al*, 1983). In mice, the maternal effect through the uterine environment or through the serum of the pregnant mice affects the frequencies of cleft lip. In the high cleft lip frequency strains, more embryos surviving with the less advanced position of the maxillary prominence and reduced epithelial activity compared with the low cleft lip frequency strain may be due to the maternal effect. More embryos surviving with smaller maxillary prominences and reduced epithelial activity may explain the deficient primary palatal and mesenchymal component areas in the high cleft lip frequency strains (A/WySn and CL/Fr) compared with the low cleft lip frequency strain (A/J).

e. Multifactorial threshold model for primary palate development

My results may be considered under the threshold model for

mesenchymal formation in the different strains of mice tested (Fig. 24). Possibly, the earlier mesenchymal replacement in noncleft lip strains provides favorable growth of the primary palate while delayed mesenchymal replacement in the low cleft lip strain (A/J) results in less favorable growth of primary palate with about 5 % cleft lip frequency. High cleft lip frequency strains have the poorest primary palate growth. Mesenchymal replacement of high cleft lip frequency strains occurs late compared with both noncleft lip and low cleft lip frequency strains, resulting in about 20 to 30% cleft lip frequency. Susceptibility to cleft lip depends on where the embryo lies in relation to the threshold. Since the mesenchymal component forms later in development in cleft lip strains compared with noncleft lip strains, this suggests that the cleft lip strain's genes put it relatively closer to the threshold.

Cleft lip is considered here as an example of a congenital malformation that is clearly multifactorial. Development of the primary palate appears to depend on adequate growth of the maxillary prominence that must provide contact of epithelia and then set up mesenchymal replacement at the epithelial seam. The facial geometry, size of lateral nasal prominence, and activity of surface epithelium are factors that must be overcome so that primary palate development can occur. These factors may act together to prevent facial prominence contact and robust mesenchymal formation. The more these factors impinge on the developmental process, the greater the severity of the cleft lip malformation.

A multifactorial/threshold model for cleft lip is modified from Fraser (1976)

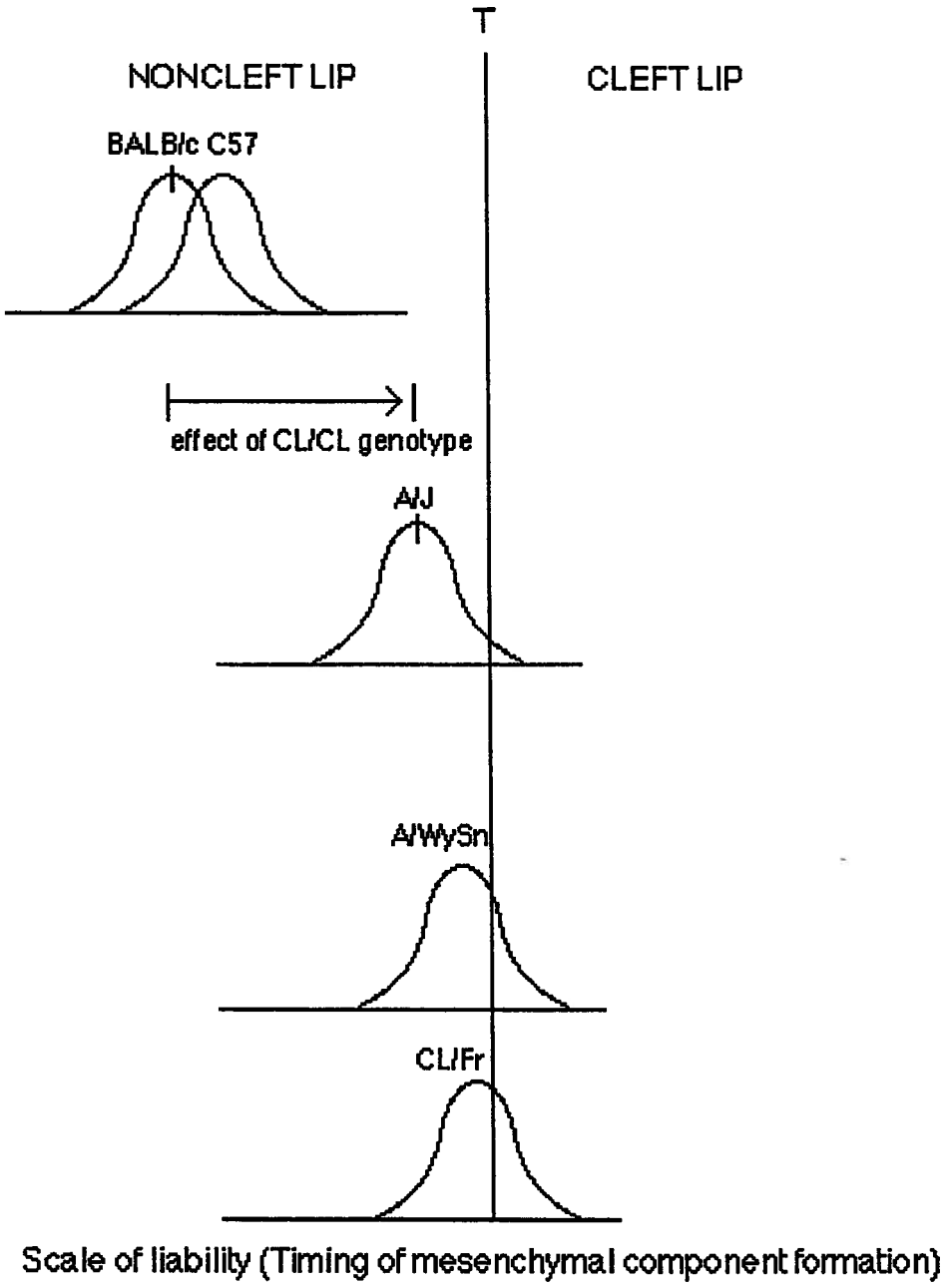


Fig. 24. A conventional multifactorial threshold showing that cleft lip may occur in some strains because mesenchymal formation is relatively late. Within strains, variation among individuals would occur due to environmental and stochastic effects, and the time of mesenchymal formation in some individuals would lie beyond some biological tolerable limit, a threshold, and in those individuals primary palate formation would fail.

and is illustrated in Figure 25. It postulates that the stage at which the mesenchyme replaces the epithelial seam and the replacement is continuously distributed. In some embryos mesenchymal formation occurs relatively early and in others relatively late. A discontinuous variable (cleft lip versus normal lip) is determined by whether a continuous variable (stage at which mesenchyme forms) puts the embryo on one side or the other of a developmental threshold (latest stage at which mesenchyme forms). Both the distribution of the variable and the threshold can be influenced by genetics and environment.

The diagram in Figure 25 illustrates the position of the distribution (relative to the threshold) as being determined primarily by the interaction of growth of the maxillary prominence, facial geometry, size of lateral nasal prominence and the activity of surface epithelium. The growth of the maxillary prominence can be influenced by other factors such as the migration of neural crest cells which may contribute to the forward growth of the maxillary prominence (Noden, 1975; Le lievre and Le Douarin, 1975). Epithelial-mesenchymal interaction is required for the growth of the maxillary prominence (Bailey *et al*, 1988; Saber *et al*, 1989). It has also been suggested that the presence of serotonin uptake sites in epithelia and serotonin binding protein in the underlying mesenchyme indicates that serotonin might be involved in epithelial-mesenchymal interactions (Lauder *et al*, 1988).

III. Primary choana formation

During vertebrate evolution, the development of primary choanae or

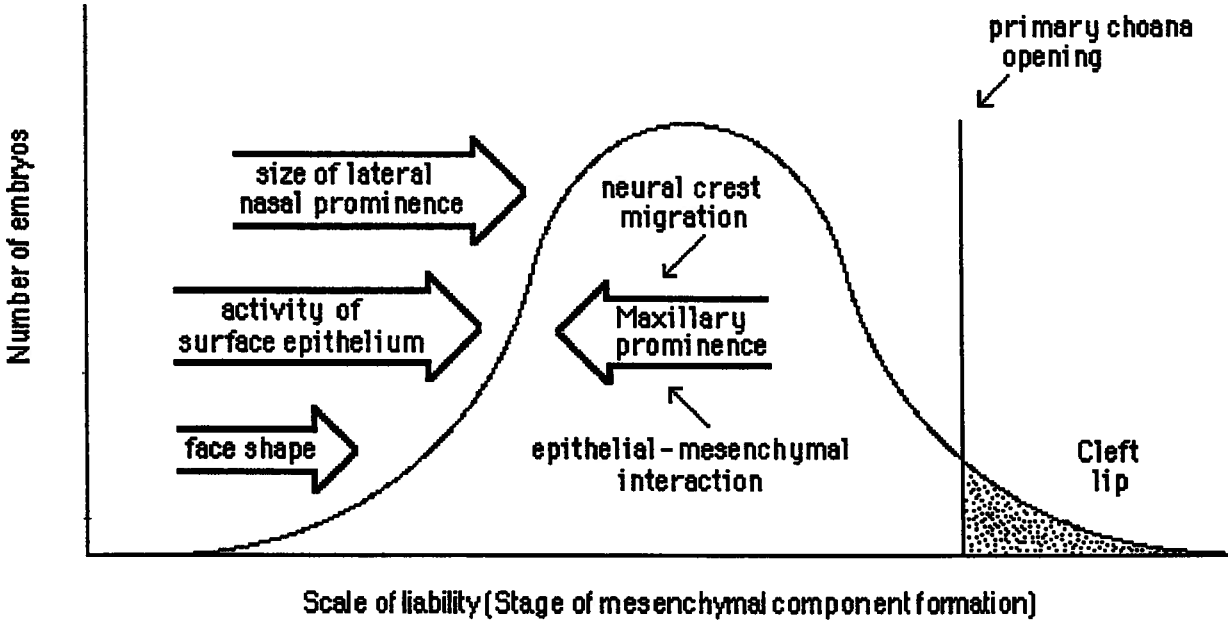


Fig. 25. A diagram illustrating the multifactorial nature of cleft lip. Stages at which the mesenchymal component forms is represented by the growth of maxillary prominence on the one side and influenced by the face shape, size of lateral nasal prominence and epithelial activity on the other side. Position of the threshold varies with the timing of primary choana opening. See text for details (Modified from Fraser, 1976).

internal nares signifies an important landmark in the adoption of an air-breathing existence (Hyman, 1942; Carter,1967). A definitive primary palate is also established as a consequence of this connection (Tamarin, 1982). Later, the elevation and fusion of the palatal shelves create the nasopharyngeal canal thereby causing the internal nares to assume a more posterior position as the secondary choanae (Hyman, 1942). Preliminary observations of primary choana formation provide a reference for definitive primary palate formation at progressive tail somite developmental stages. One noncleft (C57BL/6J) and one cleft lip strain (CL/Fr) were observed. Primary choana formation takes place at 18 to 20 tail somites in both strains. The number of embryos with primary choanae formation at 18 tail somites is significantly higher in CL/Fr (80%) than in C57BL/6J (12.5%).

Since most of the embryos in the cleft lip strain (CL/Fr) have their primary choanae formed at 18 tail somites compared with primary choanae formation at 20 tail somites in noncleft lip strain (C57BL/6J), the earlier cavitation and cleavage of the dorsal part of the nasal fin of cleft lip strain embryos may extend to the ventral part of the nasal fin which has delayed mesenchymal replacement. This earlier cleavage of the dorsal part of the nasal fin in CL/Fr than in C57BL/6J may be related to the more convergent medial nasal prominences in CL/Fr than in C57BL/6J (Trasler, 1968; Juriloff and Trasler, 1976). Streeter (1948) has shown that in normal human embryos at stage 17, the nasal fin becomes transformed from an epithelium to an epithelial-lined passage as a result of the coalescence of its cleavage spaces, which results in primary choana formation

(Fig. 26). Warbrick (1960) has suggested that the nasal fin may persist through the developmental stages and prevent the mesenchyme of maxillary and fronto-nasal prominences from making contact. Subsequently the dorsal part of the nasal fin undergoes the normal cavitation and cleavage to form the primary choana. An extension of this cavitation and cleavage into an abnormally persisting ventral part of the nasal fin would result in cleft lip formation.

The cleft lip mouse strain (CL/Fr) in this study shows both earlier primary choana formation at 18 tail somites and delayed mesenchymal component formation at 16 tail somites after the fusion of the epithelia compared to noncleft lip strain (C57BL/6J). Here primary choana formation takes place at 20 tail somites and mesenchymal penetration takes place at 13 tail somites. These two factors were addressed in the threshold model of Figure 25 as an explanation of the etiology of cleft lip from various genetic and environmental factors. The number of embryos falling beyond the threshold also varies with the position of the threshold, which in turn varies with the timing of primary choana formation. Thus the position of the threshold is also depicted as a continuous variable. In summary, the primary choana forms at a certain stage, so that if mesenchymal component formation is delayed by more than a certain critical amount, the mesenchymal component will form too late to accomplish normal primary palate formation, and a cleft lip will result. For example, in CL/Fr, after primary and secondary fusion, certain embryos have no mesenchymal component formation by 16 tail somites which may fail to allow a primary palate formation at 18 tail somites when the opening of primary choana starts.

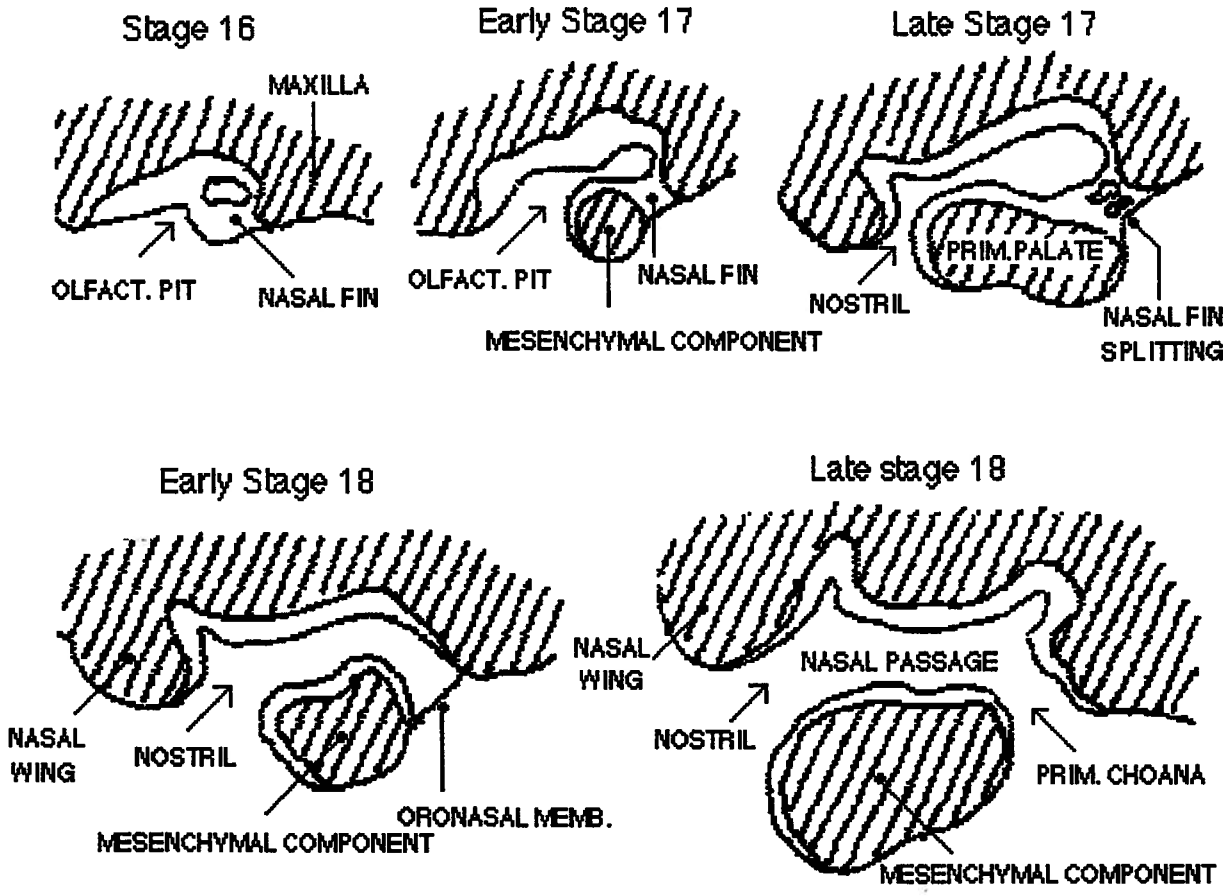


Fig. 26. Outlines of sagittal sections showing the steps in the formation of the primary palate in human embryo stage 16, 17 and 18. The platelike nasal fin is the epithelium which by characteristic splitting phenomenon produces the primary choanae (Modified From Streeter, 1948).

CONCLUSIONS

The present study of primary palate formation in mice with genetic cleft lip and mice without cleft lip at specific chronological age and tail somite number provides base line data for primary palatogenesis. In noncleft lip strains, BALB/cByJ has similar chronological age to three cleft lip strains and is considered an appropriate control for studying the cleft lip malformation. Also BALB/cByJ shares many alleles with the cleft lip strains except for the cleft lip genes. C57BL/6J is another noncleft lip strain and shows younger chronological age than both BALB/cByJ and A/- strains at the stage of primary palate development, which may make this tissue relatively "younger" than in other strains. Future work studying normal and abnormal primary palate formation should use BALB/cByJ as the primary control.

In each cleft lip strain, cleft lip frequencies and resorption rates are reciprocally related, and the difference between low cleft lip frequency strain (A/J) and high cleft lip frequency strains (A/WySn and CL/Fr) are significant. It is believed that maternal effects cause the differences in cleft lip frequencies and resorption rates for different cleft lip strains, and this maternal trait may be due to a difference in survival rate of cleft lip fetuses (Davidson *et al.* 1969; Juriloff and Fraser, 1980). It has also been shown that this maternal effect on cleft lip is mediated through uterine environment (Bornstein et al. 1970). We expect that this maternal effect on the cleft lip frequency and resorption rate affects the primary palatal structures in the form of general developmental delays and results in more deficient palatal structures in high cleft lip frequency strains (A/WySn and CL/Fr)

than in the low cleft lip frequency strain (A/J).

The ovarian site of the uterine horn has a higher cleft lip frequency than other sites within the uterus, but resorption rate is not influenced by the site within the uterus. It has been suggested that cleft lip embryos have higher survival rate at the ovarian site. Consequently, the resorption rate is lower and the cleft lip frequency is higher at the ovarian site than at other sites of the uterus (Juriloff and Fraser, 1980). My results show higher cleft lip frequency at the ovarian site compared to other sites but no difference in resorption frequency. Further studies looking at internal development of the primary palate in embryos taken from the ovarian and other sites are required to determine the morphological effects the uterine site has on development of the primary palate.

During early stages of primary palate development, forward growth of the maxillary prominence is delayed in CL/Fr compared to C57BL/6J. The possible mechanism is either delayed neural crest migration after neural tube formation, or reduced neural crest cell proliferation at the stage of induction of the nasal placode. Formation of the nasal fin represents the early fusion of facial prominences. The slower growth rate of the nasal fin in CL/Fr mice compared to C57BL/6J may be associated with delayed forward growth of the maxillary prominence and other factors such as a less divergent medial nasal prominences (Trasler, 1968). These etiologic factors may represent the biological traits of the expression of the same single gene which causes cleft lip in mice.

A further analysis of fused primary palates was undertaken at a stage during mesenchymal replacement. Noncleft lip strain mice had a larger total

primary palate area than cleft lip strains due to their genotype. Within noncleft lip strains, larger primary palate areas in BALB/cByJ than C57BL/6J are associated with different genetic backgrounds and may be affected by the younger chronological age of C57BL/6J at similar tail somite stages. The strain of low cleft lip frequency (A/J) has a larger primary palate area than strains of high cleft lip frequency (A/WySn and CL/Fr) which may be associated with depressed ability of the surface epithelium (Millicovsky *et al*, 1982; Forbes *et al*, 1989). The maternal effect on the uterine environment of the pregnant mice results in more embryos having cleft lip in A/WySn and CL/Fr compared to less embryos having cleft lip in A/J. Thus there are many embryos in A/WySn and CL/Fr with depressed activity of epithelium which results in smaller internal contact area in A/WySn and CL/Fr compared with A/J strain.

The primary palate area is also larger in A/WySn than in CL/Fr. Nevertheless they still have the same cleft lip frequency. A possible explanation is that a large portion of CL/Fr embryos have secondary fusion as reported by Millicovsky *et al* (1982). CL/Fr embryos with smaller fused areas may represent primary fusion with or without secondary fusion, compared to A/WySn which may not have secondary fusion. Eventually, these CL/Fr embryos with secondary fusion will have successful primary palate formation, and the cleft lip frequency will end up the same between A/WySn and CL/Fr.

Partial least squares analysis was applied to determine the best predictor for primary palate development. In this study, forward growth of the maxillary prominence was shown to be a better predictor for primary palate area formation

than tail somite number and body weight, especially in cleft lip strains. The delayed formation of primary palate area can be partially attributed to the delayed forward growth of the maxillary prominence.

Both the qualitative and quantitative results of mesenchymal component formation in the five strains can be divided into noncleft lip, low cleft lip and high cleft lip frequency groups as the result of the cleft lip frequency study. Thus, from either the time of formation or the size of the mesenchymal component, we can predict if the embryo belongs to a noncleft lip strain, a low or a high cleft lip frequency strain. The partial least squares analysis also shows that the position of the maxillary prominence is a better predictor than tail somite for mesenchymal component formation.

The forward growth of the maxillary prominence in the five strains studied can also be divided into noncleft lip, low and high cleft lip frequency groups which are similar to the results of the primary palatal and mesenchymal component areas study. Hence, growth of the primary palatal area, mesenchymal component and position of the maxillary prominence are specific indicators for primary palate development. Comparison of the primary palatal and mesenchymal areas at the same position of the maxillary prominence showed that both primary palatal and mesenchymal areas are still significantly different among noncleft, low and high cleft lip frequency groups, although these differences are reduced to half the differences observed in analysis of covariance with tail somite number. Delayed forward growth of the maxillary prominence may be partly associated with delayed primary palatal and mesenchymal formation in cleft lip strains. Other

etiologic factors such as less divergent medial nasal prominences may contribute to the delayed primary palatal and mesenchymal formation as well (Trasler, 1968; Juriloff and Trasler, 1976; Millicovsky *et al*, 1982).

The maternal effect on the higher survival rate of cleft lip embryos in A/WySn and CL/Fr compared with A/J may explain the less advanced position of the maxillary prominence in the high cleft lip frequency strains. This less advanced position of the maxillary prominence may partially contribute to the deficient primary palatal and mesenchymal areas in the A/WySn and CL/Fr strains compared with the A/J strain. Depressed activity of the surface epithelium may also contribute to the reduced size of the primary palatal and the mesenchymal bridge areas in A/WySn and CL/Fr than in A/J strain (Millicovsky *et al*, 1982; Forbes *et al*, 1989).

A multifactorial threshold model is suggested from this study. The stage of mesenchymal replacement is applied as a scale of liability for the cleft lip malformation. Genotypes of noncleft lip or cleft lip determine the continuous distribution toward the favorable growth of the primary palate, which is away from the threshold, or unfavorable growth of primary palate which is closer to the threshold. Both the distribution of the variable and threshold can be influenced by genetic and environmental factors. Evaluation of the hypothesis that the position of the continuous distribution is determined by the growth of the maxillary prominence will require further study of the relation between the growth of the maxillary prominence, epithelial seam replacement and the enlargement of the mesenchymal component. Future studies should focus on whether delayed

growth of the maxillary prominence is caused by delayed formation, migration, or proliferation of neural crest and/or insufficient epithelial-mesenchymal interactions. Also, it should be confirmed whether this delay in maxillary prominence growth goes on to cause insufficient mesenchymal replacement leading to various degrees of cleft lip severity.

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APPENDIX 1: The abbreviations applied in the Appendix 2 and 3.

TS: Tail somites.

HR: Age in hours.

WT: Body weight in mg.

RMXP:Maxillary prominence depth of right side in μm

RPP: Primary palate area of right side in μm^2.

RM: Mesenchymal area of right side in μm^2.

RPD: Primary palate depth of right side in μm.

RPH: Maximal primary palate height of serial sections of right side in μm.

RMH: Maximal mesenchymal height of serial sections of right side in μm.

LMXP:Maxillary prominence depth of left side in μm

LPP: Primary palate area of left side in μm^2.

LM: Mesenchymal area of left side in μm^2.

LPD: Primary palate depth of left side in μm.

LPH: Maximal primary palate height of serial sections of left side in μm.

LMH: Maximal mesenchymal height of serial sections of left side in μm.

APPENDIX 2

BALB/cBYJ

OBS	TS	HR	WT	RMXP	LMXP	RPP	LPP	RM	LM	RPD	LPD	RPH	LPH	RMH	LMH
1	13	277	22	140	140	29086	30664	0	0	245	245	151.9	156.8	0.0	0.0
2	13	277	31	189	189	44384	43286	3292	4527	301	287	215.6	205.8	58.8	68.6
3	13	277	22	161	168	42051	41983	3361	3567	259	259	210.7	220.5	68.6	73.5
4	13	277	22	182	168	39719	40679	2469	411	273	287	196.0	186.2	49.0	29.4
5	14	276	26	196	189	58584	52410	9741	9192	301	301	274.4	245.0	98.0	127.4
6	14	276	32	217	210	49735	54811	6242	11792	301	315	225.4	235.2	112.7	117.6
7	14	275	31	168	175	46030	52067	7957	13102	287	301	210.7	235.2	102.9	127.4
8	14	274	32	175	175	45619	44864	5968	7271	287	287	215.6	205.8	88.2	88.2
9	14	275	31	182	196	45207	47196	12073	9947	287	301	205.8	225.4	117.6	102.9
10	14	275	30	210	189	57349	54331	11936	14187	343	329	220.5	215.6	117.6	127.4
11	14	275	32	210	210	54125	58310	15778	13171	315	343	235.2	225.4	137.2	127.4
12	15	276	27	217	217	55703	58035	16052	13857	301	315	264.6	254.8	156.8	137.2
13	15	281	32	210	196	46373	45687	13857	15435	287	301	235.2	220.5	137.2	147.0
14	15	277	29	231	210	60093	56457	17767	13582	315	322	274.4	245.0	156.8	156.8
15	15	274	35	224	224	50215	57761	12279	18727	301	301	245.0	264.6	147.0	171.5
16	15	276	37	224	203	59819	60093	24901	24421	329	343	254.8	249.9	176.4	171.5
17	15	276	33	231	231	60162	56869	19070	17493	343	329	264.6	245.0	156.8	147.0
18	15	276	33	224	203	60299	59956	20991	23529	329	329	254.8	245.0	161.7	161.7
19	16	276	36	224	210	59819	60642	18659	19894	315	315	274.4	294.0	176.4	186.2
20	16	277	28	231	224	60848	53576	17355	13514	329	315	259.7	245.0	161.7	132.3
21	16	277	40	245	259	67502	70109	25176	30321	357	371	279.3	274.4	176.4	176.4
22	16	278	37	245	245	63504	65699	27283	27910	357	357	240.1	245.0	166.6	166.6

C57BL/6J

OBS	TS	HR	WT	RMXP	LMXP	RPP	LPP	RM	LM	RPD	LPD	RPH	LPH	RMH	LMH
1	13	264	24	154	154	21746	22569	274	343	196	210	137.2	137.2	19.6	24.5
2	13	264	21	161	154	23461	24078	137	0	217	231	127.4	137.2	9.8	0.0
3	13	264	26	161	168	27920	28812	2195	137	245	259	147.0	147.0	39.2	9.8
4	13	264	26	203	182	35191	35603	4870	8575	287	280	161.7	176.4	68.6	98.0
5	13	264	23	182	182	27097	31007	411	8575	245	252	151.9	161.7	29.4	98.0

6	14	264	26	224	210	40954	40817	10427	12485	301	301	200.9	205.8	117.6	112.7
7	14	272	27	196	196	32516	39513	5831	8369	273	315	147.0	156.8	58.8	78.4
8	14	264	27	175	161	26273	23735	1303	548	259	245	137.2	132.3	39.2	24.5
9	14	264	25	203	189	41297	32104	10015	4870	301	287	205.8	156.8	102.9	78.4
10	14	264	25	196	196	36838	38347	8712	10152	273	301	210.7	181.3	117.6	102.9
11	14	264	31	203	203	42326	42120	13788	20923	301	287	215.6	215.6	137.2	171.5
12	14	264	28	182	210	35123	38759	12348	9741	259	287	186.2	205.8	127.4	122.5
13	15	272	34	224	238	45687	49186	16464	19070	371	371	166.6	176.4	117.6	132.3
14	15	272	38	238	266	56114	54468	25587	23598	343	357	225.4	205.8	156.8	166.6
15	15	272	34	224	238	48568	51930	17973	20099	329	329	205.8	215.6	137.2	147.0
16	15	272	35	196	168	42394	41846	12759	13651	329	329	166.6	156.8	98.0	98.0
17	15	264	38	252	238	41091	48157	12005	20991	301	329	196.0	205.8	117.6	147.0
18	15	264	25	224	224	41297	39513	9192	11593	273	287	225.4	205.8	117.6	112.7
19	15	264	27	238	224	42943	44178	13720	14680	273	287	245.0	225.4	156.8	147.0
20	16	270	33	252	252	41640	37112	20099	21814	273	287	205.8	205.8	156.8	156.8
21	16	270	39	252	252	51998	55291	23461	30115	273	287	274.4	294.0	196.0	215.6
22	16	270	36	280	280	58035	59887	27234	30115	301	343	284.2	254.8	196.0	196.0
23	16	275	26	210	210	44521	50078	19276	24696	315	329	196.0	215.6	137.2	166.6
24	16	264	32	252	252	46510	45207	20442	21128	273	301	254.8	245.0	176.4	176.4
25	16	270	37	238	238	45550	49254	18522	22363	329	287	245.0	254.8	166.6	186.2
26	16	272	28	238	238	40405	46785	8918	14954	259	301	196.0	215.6	98.0	137.2

A/J

OBS	TS	HR	WT	RMXP	LMXP	RPP	LPP	RM	LM	RPD	LPD	RPH	LPH	RMH	LMH
1	13	276	21	105	98	22706	16807	0	0	175	154	181.3	161.7	0.0	0.0
2	13	277	22	119	119	27302	20785	0	0	231	189	176.4	156.8	0.0	0.0
3	13	276	26	154	147	25587	31899	0	1166	217	252	166.6	176.4	0.0	29.4
4	13	277	23	140	140	25862	23529	0	0	252	245	147.0	147.0	0.0	0.0
5	14	276	26	147	133	35809	29292	1783	0	245	217	225.4	200.9	68.6	0.0
6	14	281	23	133	147	22363	25176	548	0	217	231	137.2	0.0	19.6	0.0
7	14	277	27	168	175	30115	28674	411	1234	273	259	166.6	166.6	29.4	49.0
8	14	277	24	161	161	23735	24764	548	0	245	245	147.0	147.0	39.2	0.0

9	14	277	24	161	147	36289	29155	3292	686	287	273	191.1	156.8	73.5	29.4
10	14	277	26	126	119	22912	18727	0	0	224	217	166.6	137.2	0.0	0.0
11	14	275	26	154	140	29841	32036	0	0	259	273	166.6	166.6	0.0	0.0
12	15	277	24	175	154	36563	30595	1646	2949	273	259	196.0	176.4	49.0	68.6
13	15	275	26	182	161	44795	37798	8712	4596	343	315	196.0	186.2	88.2	78.4
14	15	276	33	189	175	38964	39033	5145	3773	259	273	225.4	210.7	107.8	78.4
15	15	277	22	189	175	39170	43355	1097	5831	329	315	181.3	205.8	44.1	98.0
16	15	277	22	196	182	46922	40611	3567	2332	329	315	205.8	200.9	88.2	68.6
17	15	281	36	196	189	29498	33751	480	343	245	280	176.4	166.6	34.3	24.5
18	15	276	36	175	189	37592	43561	9809	12073	259	287	225.4	235.2	132.3	132.3
19	15	276	32	196	182	44864	42875	9809	9123	315	294	220.5	215.6	127.4	107.8
20	16	277	29	245	231	58104	49392	18727	9398	357	343	254.8	240.1	142.1	122.5
21	16	277	29	224	231	56800	50695	20717	16258	343	343	235.2	240.1	171.5	161.7
22	16	277	25	196	196	38004	45207	8575	7683	259	287	235.2	235.2	137.2	137.2
23	16	277	29	231	231	45481	46853	10084	15503	343	329	225.4	225.4	122.5	156.8
24	16	281	30	196	203	38553	48020	7477	9809	287	315	196.0	220.5	102.9	156.8
25	16	279	30	112	126	617	1029	0	0	49	56	19.6	39.2	0.0	0.0

A/WySn

OBS	TS	HR	WT	RMXP	LMXP	RPP	LPP	RM	LM	RPD	LPD	RPH	LPH	RMH	LMH
1	13	279	23	126	126	21197	17287	0	0	189	175	166.6	151.9	0.0	0.0
2	13	279	16	70	56	2881	4939	0	0	77	175	63.7	44.1	0.0	0.0
3	13	281	22	112	112	27714	17424	0	0	203	210	132.3	147.0	0.0	0.0
4	13	279	18	119	112	12553	12553	0	0	161	175	102.9	102.9	0.0	0.0
5	13	280	20	98	105	11250	15435	0	0	161	189	98.0	122.5	0.0	0.0
6	14	272	29	133	119	19756	20854	0	0	287	259	98.0	132.3	0.0	0.0
7	14	272	28	119	133	25990	24901	0	274	287	259	127.4	142.1	0.0	19.6
8	14	280	28	154	140	29292	19413	0	0	273	252	166.6	117.6	0.0	0.0
9	14	279	25	182	175	32379	30664	1372	2881	259	245	196.0	191.1	49.0	73.5
10	14	281	24	140	161	27988	26959	0	0	280	273	127.4	147.0	0.0	0.0
11	14	280	23	112	126	19413	19756	0	0	231	231	117.6	122.5	0.0	0.0
12	15	279	26	182	189	35054	33545	0	3224	301	287	181.3	196.0	0.0	0.0

13	15	276	27	189	182	37867	30870	2401	2538	329	287	196.0	171.5	58.8	53.9
14	15	276	27	98	126	26136	14131	0	0	259	245	171.5	137.2	0.0	0.0
15	15	272	33	175	154	34505	29155	754	0	329	273	147.0	147.0	34.3	0.0
16	15	278	26	168	147	30046	26273	0	274	259	231	166.6	181.3	0.0	19.6
17	15	278	30	175	182	37935	42943	1029	3978	301	315	186.2	205.8	39.2	78.4
18	16	272	29	224	231	45207	43355	11113	6997	301	315	225.4	230.3	127.4	98.0
19	16	272	26	189	189	38347	39650	2812	754	301	315	215.6	191.1	83.3	34.3
20	16	281	28	189	182	40611	37387	3361	2058	301	329	205.8	181.3	73.5	58.8
21	16	281	33	203	196	36495	32379	0	0	287	273	220.5	166.6	0.0	0.0
22	16	272	28	210	203	41503	39582	6791	6997	266	259	235.2	240.1	102.9	117.6

CL/Fr

OBS	TS	HR	WT	RMXP	LMXP	RPP	LPP	RM	LM	RPD	LPD	RPH	LPH	RMH	LMH
1	13	278	27	56	77	823	1029	0	0	21	49	49.0	29.4	0.0	0.0
2	13	272	36	154	91	29909	10770	0	0	301	189	137.2	68.6	0.0	0.0
3	13	272	26	91	49	5968	5488	0	0	133	133	73.5	68.6	0.0	0.0
4	13	281	36	147	147	20305	18247	0	0	231	203	122.5	122.5	0.0	0.0
5	13	280	24	189	182	39856	34643	6174	0	329	329	181.3	151.9	78.4	0.0
6	13	278	24	98	105	12210	23529	0	0	189	245	88.2	132.3	0.0	0.0
7	13	281	26	84	91	13445	14680	0	0	189	203	93.1	83.3	0.0	0.0
8	14	281	37	112	98	11113	7408	0	0	147	161	117.6	68.6	0.0	0.0
9	14	277	25	168	168	29772	23255	411	0	301	273	137.2	122.5	19.6	0.0
10	14	280	28	42	63	686	1509	0	0	7	21	49.0	78.4	0.0	0.0
11	14	279	27	126	119	17561	16395	0	0	315	259	88.2	88.2	0.0	0.0
12	14	280	32	168	105	30321	24010	0	0	301	273	132.3	122.5	0.0	0.0
13	14	278	29	105	126	5076	1097	0	0	161	35	58.8	39.2	0.0	0.0
14	14	279	27	126	133	26273	21609	0	0	287	245	122.5	117.6	0.0	0.0
15	14	282	36	140	140	18796	24696	0	0	217	245	117.6	137.2	0.0	0.0
16	14	280	29	168	182	33957	40748	0	0	301	315	166.6	176.4	0.0	0.0
17	14	272	36	168	119	25450	17493	0	0	203	161	166.6	137.2	0.0	0.0
18	15	281	26	140	161	25862	25930	0	0	266	252	147.0	156.8	0.0	0.0

19	15	278	36	189	154	21814	14200	0	0	273	217	122.5	98.0	0.0	0.0
20	15	281	29	112	98	15503	11936	0	0	217	189	98.0	88.2	0.0	0.0
21	15	278	24	154	119	17561	5625	0	0	161	77	181.3	107.8	0.0	0.0
22	15	277	33	168	147	28194	3704	0	0	315	147	142.1	49.0	0.0	0.0
23	16	282	40	210	175	27302	31830	0	686	273	301	156.8	166.6	0.0	49.0
24	16	282	34	182	196	33819	35054	274	1234	273	259	181.3	196.0	19.6	49.0
25	16	278	43	210	189	29635	33888	2675	5968	259	273	186.2	196.0	68.6	88.2
26	16	280	32	182	182	40885	28263	5076	0	329	301	176.4	137.2	78.4	0.0
27	16	279	33	189	203	38416	36358	5968	10976	301	301	186.2	196.0	83.3	98.0
28	16	277	34	189	168	39925	32928	1852	0	343	315	156.8	147.0	53.9	0.0
29	16	280	30	98	91	2744	12622	0	0	175	161	49.0	117.6	0.0	0.0
30	16	281	33	196	210	42326	35603	1234	0	287	287	215.6	196.0	88.2	0.0
31	16	279	31	210	231	42875	33751	11936	2195	301	301	230.3	176.4	137.2	78.4
32	16	277	30	105	161	7340	13445	0	0	161	203	98.0	98.0	0.0	0.0
33	16	281	38	217	224	32585	41983	4664	7408	259	315	205.8	215.6	88.2	117.6
34	16	280	27	210	196	40542	36083	12279	2332	287	301	205.8	176.4	127.4	49.0
35	16	281	22	105	112	16669	19276	0	0	217	273	98.0	107.8	0.0	0.0
36	16	278	31	196	196	33133	42532	1989	12965	287	343	166.6	176.4	83.3	107.8
37	16	278	29	182	182	27028	30046	0	891	273	273	147.0	166.6	0.0	39.2

APPENDIX 3

BALB/cByJ
PEARSON CORRELATION COEFFICIENTS / N = 22

	TS	WT	RMXP	RPP	RM	RPD	RPH	RMH
TS	1.00	0.69	0.85	0.81	0.88	0.74	0.77	0.90
WT		1.00	0.69	0.64	0.74	0.73	0.53	0.74
RMXP			1.00	0.90	0.86	0.88	0.83	0.90
RPP				1.00	0.88	0.92	0.90	0.89
RM					1.00	0.85	0.75	0.92
RPD						1.00	0.70	0.79
RPH							1.00	0.85
RMH								1.00

	TS	WT	LMXP	LPP	LM	LPD	LPH	LMH
TS	1.00	0.69	0.83	0.80	0.86	0.72	0.80	0.85
WT		1.00	0.73	0.78	0.87	0.77	0.66	0.79
LMXP			1.00	0.88	0.82	0.85	0.77	0.80
LPP				1.00	0.91	0.92	0.86	0.90
LM					1.00	0.87	0.77	0.90
LPD						1.00	0.65	0.78
LPH							1.00	0.91
LMH								1.00

C57BL/6J
PEARSON CORRELATION COEFFICIENTS / N = 26

	TS	WT	RMXP	RPP	RM	RPD	RPH	RMH
TS	1.00	0.68	0.84	0.79	0.83	0.49	0.74	0.82
WT		1.00	0.71	0.76	0.79	0.64	0.58	0.71
RMXP			1.00	0.86	0.83	0.52	0.84	0.86
RPP				1.00	0.95	0.74	0.85	0.92
RM					1.00	0.65	0.83	0.94
RPD						1.00	0.37	0.57
RPH							1.00	0.93
RMH								1.00

	TS	WT	LMXP	LPP	LM	LPD	LPH	LMH
TS	1.00	0.68	0.81	0.78	0.83	0.59	0.77	0.81
WT		1.00	0.72	0.77	0.79	0.63	0.63	0.72
LMXP			1.00	0.87	0.87	0.67	0.83	0.87
LPP				1.00	0.92	0.83	0.82	0.89
LM					1.00	0.69	0.87	0.96
LPD						1.00	0.41	0.62
LPH							1.00	0.92
LMH								1.00

A/J
PEARSON CORRELATION COEFFICIENTS / N = 25

	TS	WT	RMXP	RPP	RM	RPD	RPH	RMH
TS	1.00	0.51	0.72	0.47	0.68	0.32	0.26	0.74
WT		1.00	0.42	0.17	0.40	0.03	0.18	0.45
RMXP			1.00	0.86	0.78	0.81	0.69	0.83
RPP				1.00	0.80	0.93	0.89	0.83
RM					1.00	0.61	0.64	0.90
RPD						1.00	0.79	0.65
RPH							1.00	0.74
RMH								1.00

	TS	WT	LMXP	LPP	LM	LPD	LPH	LMH
TS	1.00	0.51	0.76	0.56	0.71	0.41	0.37	0.77
WT		1.00	0.49	0.33	0.45	0.20	0.29	0.41
LMXP			1.00	0.86	0.84	0.80	0.62	0.87
LPP				1.00	0.79	0.94	0.80	0.87
LM					1.00	0.64	0.66	0.94
LPD						1.00	0.71	0.72
LPH							1.00	0.73
LMH								1.00

A/WySn
PEARSON CORRELATION COEFFICIENTS / N = 22

	TS	WT	RMXP	RPP	RM	RPD	RPH	RMH
TS	1.00	0.74	0.83	0.85	0.59	0.74	0.83	0.68
WT		1.00	0.69	0.77	0.27	0.88	0.63	0.34
RMXP			1.00	0.91	0.64	0.74	0.88	0.73
RPP				1.00	0.60	0.85	0.91	0.70
RM					1.00	0.31	0.60	0.92
RPD						1.00	0.65	0.43
RPH							1.00	0.70
RMH								1.00

	TS	WT	LMXP	LPP	LM	LPD	LPH	LMH
TS	1.00	0.74	0.84	0.83	0.56	0.84	0.74	0.57
WT		1.00	0.68	0.68	0.30	0.75	0.59	0.31
LMXP			1.00	0.94	0.72	0.81	0.91	0.68
LPP				1.00	0.72	0.87	0.91	0.74
LM					1.00	0.50	0.78	0.90
LPD						1.00	0.68	0.52
LPH							1.00	0.76
LMH								1.00

CL/Fr
PEARSON CORRELATION COEFFICIENTS / N = 37

	TS	WT	RMXP	RPP	RM	RPD	RPH	RMH
TS	1.00	0.28	0.54	0.42	0.36	0.34	0.50	0.49
WT		1.00	0.49	0.26	0.01	0.21	0.34	0.15
RMXP			1.00	0.89	0.53	0.80	0.90	0.64
RP				1.00	0.59	0.86	0.91	0.70
RM					1.00	0.35	0.62	0.90
RPD						1.00	0.67	0.43
RPH							1.00	0.73
RMH								1.00

	TS	WT	LMXP	LPP	LM	LPD	LPH	LMH
TS	1.00	0.28	0.64	0.50	0.42	0.43	0.58	0.55
WT		1.00	0.32	0.24	0.28	0.17	0.30	0.40
LMXP			1.00	0.83	0.49	0.73	0.82	0.64
LPP				1.00	0.53	0.91	0.94	0.63
LM					1.00	0.39	0.52	0.89
LPD						1.00	0.78	0.46
LPH							1.00	0.67
LMH								1.00