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Abnormal development of the notochord and perinotochordal sheath in duplicitas posterior, patch and tail-short mice

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Summary. Interest in developmental interactions involving the notochord and perinotochordal sheath led to a comparative investigation of these structures in three mouse mutants. Alcian blue or periodic acid-Schiff staining of 9 1/2-13 days' gestational age embryos revealed a supernumerary notochordal-like mass of cells or a deflected notochord in association with duplication of the neural tube in mice of the duplicitas posterior stock. The perinotochordal sheath and basement membrane of the accessory notochordal masses were frequently defective. Patch and Tail-short embryos were also utilized for study by means of light microscopy using Alcian blue staining. In Patch embryos, although the notochord was sometimes compressed dorso-ventrally, it had an intact perinotochordal sheath and a defined, but undulated, basement membrane. Mesenchymal cells between the notochord and neural tube were occasionally replaced by cell-free space. In contrast, in Tail-short embryos a poorly formed, lightly staining or totally absent notochordal sheath was revealed. Indeed, it was sometimes difficult to distinguish the notochord from surrounding mesenchymal cells. In both the Patch and Tail-short embryos the notochord was also deflected from its medial position. In the three mutants studied, the direct or indirect effect of gene action appeared to be on the notochord and perinotochordal sheath, and the important role of these structures in abnormal axial development was established.

Key words: Notochord, Perinotochordal sheath, Mutant mice

Introduction

While the role of chordamesodermal cells in animal development has been under investigation since the classic experiment of Spemann and Mangold (1924), the specific role of these cells in development remains one of the unresolved questions of biology. Three groups of mice which exhibit axial anomalies were utilized to investigate the inadequately understood role of the notochord, perinotochordal sheath and associated mesenchymal cells.

A group of duplication anomalies («hereditary doubling») found in the mouse was first described some sixty years ago (Danforth, 1925, 1930) but to date these defects have been incompletely investigated. We have termed these abnormalities the «duplicitas posterior syndrome» and have described four major types of manifestations (Center, 1969). These are 1) reduced hind-limb development, 2) supernumerary hind-limb combined with reduced hind-limb development, 3) supernumerary hind-limbs, and 4) kinked tail. Abnormalities of the skeletal elements in the caudal region and of the urogenital tract and hindgut also occur in this manifestation. The genetic basis remains uncertain, but it is probably multigenic (Center, 1969).

One mutant used in our investigation was Patch (symbol *Ph*) which is due to a semi-dominant gene (Grüneberg and Truslove, 1960). This gene arose as a spontaneous mutation in the C57BL strain maintained by the Glaxo Laboratories. Areas of pigmented and of white fur are clearly evident in the Patch heterozygote. The *Ph/Ph* homozygote is not viable, mainly due to the presence of a large amount of hydrops. Anomalies characteristic of this group include 1) a «wavy» neural tube, 2) poorly developed vertebral column, particularly in the cervical region, and 3) blebs in the areas of the face, fore- and hind-limbs, neck, shoulders, and haunches.

Another mutant studied was Tail-short (*Ts*) which is also a semi-dominant trait. Morgan (1950) discovered Tail-short in the BALB/c inbred strain. It is lethal in the homozygous condition as illustrated by ratios obtained from crosses conducted between *Ts*/+ X *Ts*/+ (Deol, 1961). Morgan described its mode of inheritance and the

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morphology of the heterozygote. Further study of the anatomy of Tail-short revealed the details of the various skeletal abnormalities (Deol, 1961). These are 1) a short and kinked (occasionally curled) tail, 2) broader snout size in proportion to body size, 3) fused and abnormal (gaps in the arch or the centrum) vertrebrae, and 4) disproportionate length of fore- and hind-limbs. Both Patch and Tail-short embryos were kindly provided by M.S. Deol and G.M. Truslove of University College, London.

Abnormalities of the neural tube and the notochord were observed in all three lines of mice. Anomalies were seen in the tail region of the Tail-short (*Ts*) mice as early as the eleventh day (Deol, 1961). Blebs which are found in many areas of the Patch (*Ph*) body occur in the region of the notochord, and interfere with the development of the vertebrae (Grüneberg, 1963). Becuase of interest in developmental interactions among the neural tube, notochord and mesenchymal cells (Hay and Meier, 1974; Jurand, 1974; Faris and Crowe, 1975; Jacobson, 1978; Morris and Solursh, 1978; Takaya, 1978; Carlson, 1979; Youn and Malacinski, 1981; Wilson et al., 1982) the current analysis was undertaken to assay specific gene action on the developing notochord in duplicitas posterior, Patch and Tail-short mice. A normal pattern of notochordal development had been established previously in mouse embryos of nine to fourteen days (Paavola et al., 1980; Center et al., 1982).

Materials and methods

Pregnancies were timed by use of the vaginal plug method (vaginal plug = day zero) and correlation was made with Grüneberg's (1943) morphological staging. The embryos were fixed in 10% neutral buffered formalin, or occasionally in 10% unbuffered formalin. The specimens were then embedded in paraplast at 56-59° C. Serial sections of the 9 1/2 - 13 day embryos were cut at 10 μm. In order to delineate the perinotochordal sheath as clearly as possible, these were stained with modification of the periodic acid-Schiff (PAS) method developed by McManus and detailed by Pearse (1968) in which no counterstain is used, or, with Alcian blue at a pH of 2.5 (Bancroft, 1975) or at a pH of 5.8 (Chayen et al., 1973) both with or without a hematoxylin counterstain. All Patch and Tail-short embryos were subjected to Alcian blue (pH 2.5) and hematoxylin staining. The latter stain was utilized to provide adequate contrast so as to better delineate the perinotochordal sheath which stains readily with Alcian blue due to the presence of glycosaminoglycans. Although attention was focused on the caudal region, serial sections throughout the length of the trunk were also examined by means of light microscopy. In addition, three duplicitas posterior embryos at 11 days of gestation were processed by fixation in cold (4 °C) half-strength Karnovsky's solution (1965) for two hours, rinsed in 0.1M cacodylate buffer (pH 7.2), post-fixed in 1% osmium tetroxide-0.1M cacodylate buffer in the cold for 1-2 hours, dehydrated, and embedded in Epon-Araldite. Thick sections (1-3 μm) were obtained, stained with methylene blue-azure II and viewed by means of light microscopy.

Results

Of the 80 embryos examined in this current study, 21 of the 36 duplicitas posterior mice were abnormal, 5 of the 24 Patch stock embryos were abnormal, and 10 of the 20 Tail-short were abnormal.

Of the abnormal duplicitas posterior embryos, three had both an accessory neural tube and a supernumerary notochordal-like mass of cells. Four embryos exhibited an extra neural tube, and in one of the four there was also an apparent nest of additional notochordal cells. However, in four embryos, one of which was grossly normal, two notochordal structures (or one abnormal notochord) were evident, but there was no indication of duplication of the neural tube in these mice. A number of remaining embryos with gross manifestations typical of the duplicitas posterior syndrome showed no duplications of either of the above axial structures. With two exceptions, all duplications of notochordal and neural tube structures were located in the posterior end of the animals examined. All sections discussed below involved the trunk and tail regions.

The notochord appeared to be deflected from the normal position in a few embryos that showed posterior duplication anomalies grossly. Instead of being in the usual position mid-ventral to the neural tube, the notochord could be seen in a position lateral to the midline of the body (Fig. 1). In a number of cases, the basement membrane or the surrounding fibrous sheath or both elements of the perinotochordal sheath appeared to be abnormal in the supernumerary or deflected notochord when comparison was made with the notochord of a normal embryo (Figs. 2, 3). In some abnormal embryos, one of the two notochords was somewhat larger than the other (Figs. 4, 5). The notochord which was in the normal position mid-ventral to the neural tube frequently showed more complete development of the perinotochordal sheath than the one which was not in the normal position. In Epon embedded sections of two 11-day duplicitas embryos there was little indication of a sheath associated with the additional notochordal tissue. An abnormal grouping of surrounding mesenchymal cells was sometimes evident in the vicinity of the defective notochord (Figs. 2, 5). In one 13-day old embryo, while an extra neural tube was obvious, only one notochordal structure could definitely be identified. However, two concentric swirls of mesenchymal cells were observed. One of the two juxtaposed swirls had an obvious notochordal structure, which was deflected from the midline while none could be found in the adjacent swirl (Fig. 6). None of the 9 1/2 or 10-day duplicitas posterior stock embryos examined showed duplication or deflection of the notochord.

Duplication of the tail gut was confirmed as an isolated anomaly in the duplicitas posterior syndrome. However, the supernumerary tail gut was definitely found in association with duplication of the neural tube

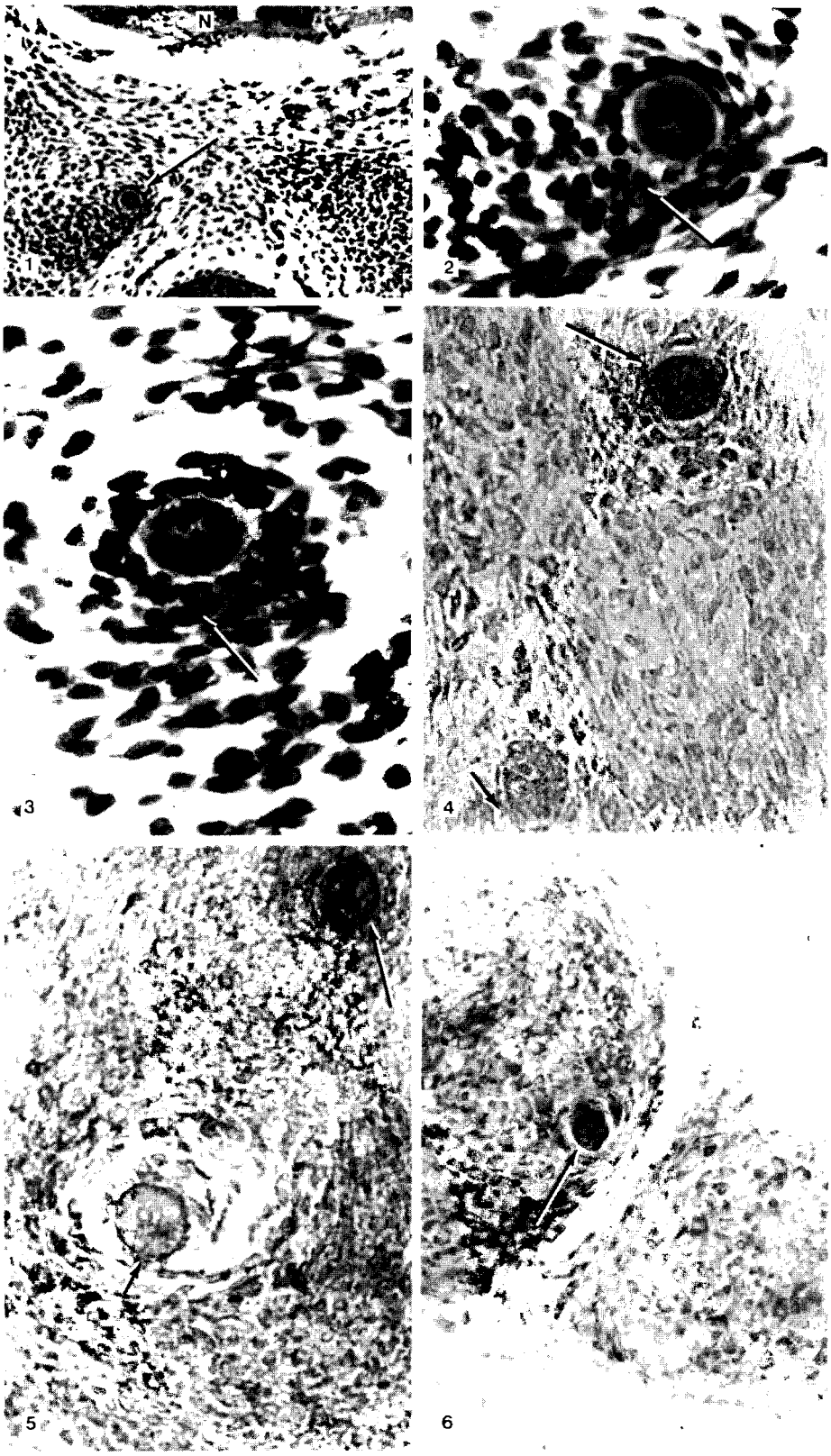

Fig. 1. Posterior trunk region of an 11-day duplicitas posterior embryo showing the notochord (arrow) deflected laterally from the normal position mid-ventral to the neural tube (N). × 140

Fig. 2. Higher magnification of notochord shown in Fig. 1. Note irregular grouping of surrounding mesenchymal cells (arrow) and adjacent defect in notochordal sheath. × 590

Fig. 3. Posterior trunk notochord of an 11-day normal. Orderly arrangement of surrounding mesenchymal cells (arrow) and normal perinotochordal sheath development. × 615

Fig. 4. Posterior trunk section of an 11-day abnormal duplicitas posterior. Two notochordal masses: one (long arrow) in the usual mid-ventral position has relatively normal sheath development; the other (short arrow) has an abnormal perinotochordal sheath. × 590

Fig. 5. Posterior trunk section of a 12-day duplicitas posterior embryo with two notochordal masses: more normal development of the perinotochordal sheath (long arrow), very little evidence of sheath and abnormal grouping of surrounding mesenchymal cells (short arrow). × 640

Fig. 6. Posterior trunk section of a 13-day duplicitas posterior embryo with a duplicate neural tube and two concentric swirls of mesenchymal cells. One swirl contains a notochord (arrow). × 400

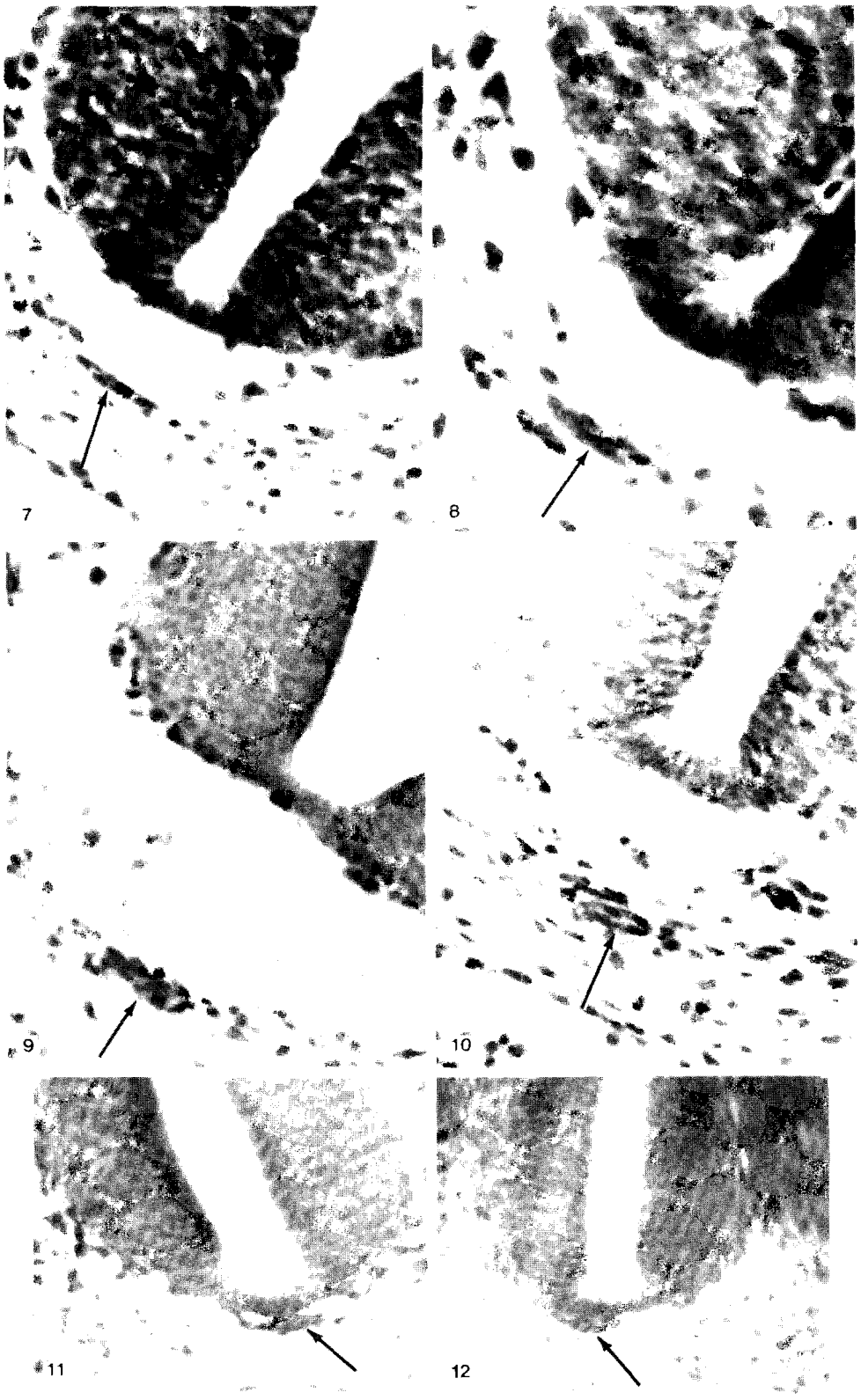

Fig. 7. Fore-limb level of a 9 1/2 day Patch embryo. The perinotochordal sheath is normal but the notochord is compressed dorso-ventrially (arrow). Cell-free space is evident between the neural tube and the notochord. × 400

Fig. 8. More anterior section of embryo shown in Fig. 7. Well-developed perinotochordal sheath on the ventral surface of the notochord (arrow). × 640

Fig. 9. Abnormal 10 1/2 day Patch embryo at the posterior trunk level. Note compressed notochord (arrow) and large amount of cell-free space between the notochord and neural tube. × 500

Fig. 10. Fore-limb level of a 10 day abnormal Patch embryo. The perinotochordal sheath, although continuous, is undulated (arrow). Increased amount of cell-free space is evident between the notochord and neural tube. × 400

Fig. 11. Posterior trunk level of a 10 day Patch embryo. The perinotochordal sheath (arrow), although continuous, is undulated. × 400

Fig. 12. More posterior section of the embryo in Fig. 11. The perinotochordal sheath (arrow) shows normal development. Basement membrane is smooth and continuous. × 400

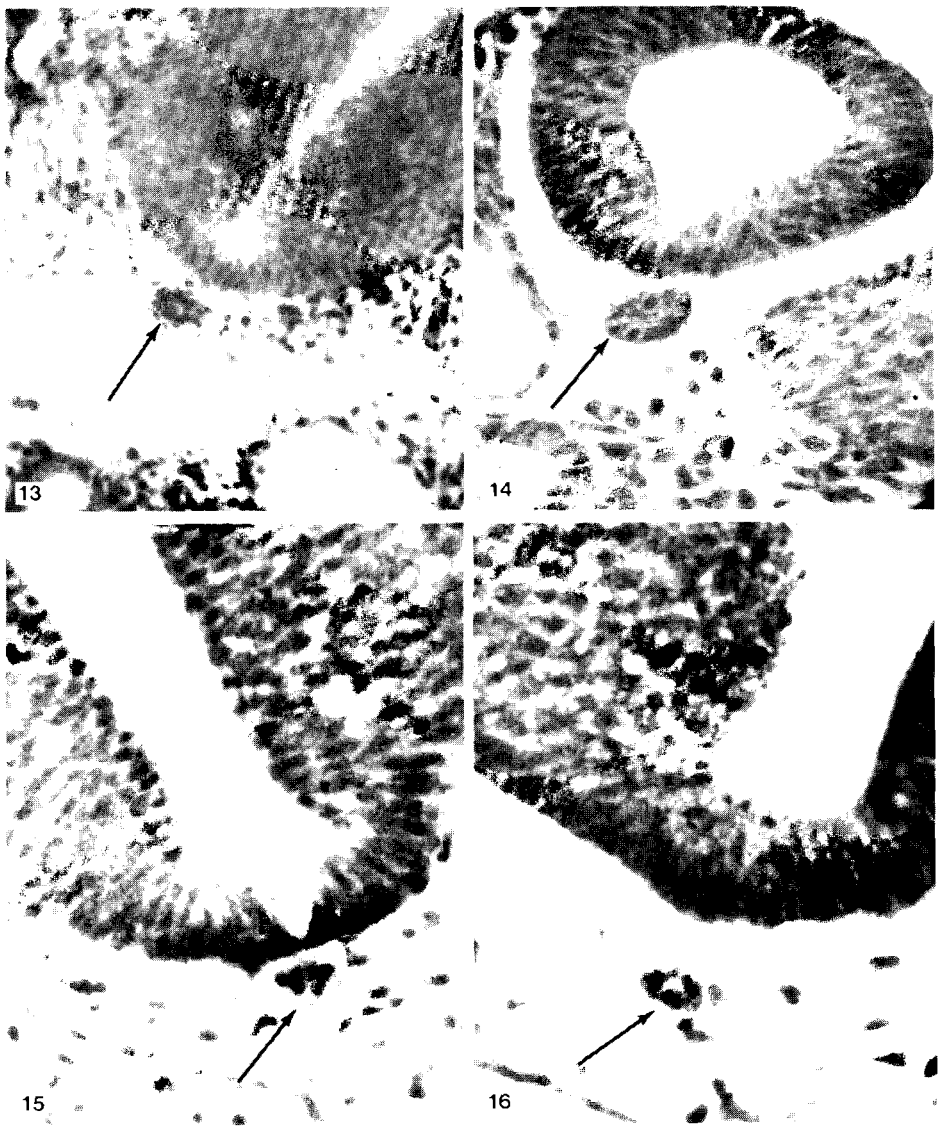

Fig. 13. Posterior trunk level of a 9 1/2 day Tail-short embryo. The perinotochordal sheath (arrow) has undergone almost total disintegration with no well-defined structure remaining. The Alcian blue staining appears faint and ill-defined. × 350 approx.

Fig. 14. Posterior trunk level of a 10 day normal embryo. Development of the notochord is normal with well-defined perinotochordal sheath (arrow). × 480 approx.

Fig. 15. Anterior trunk section of a 9 1/2 day Tail-short embryo. Abnormal development of the notochord (arrow). It is difficult to distinguish notochordal cells from mesenchymal cells. × 400

Fig. 16. Anterior trunk level of a 9 1/2 day normal embryo. Normal development of the notochord (arrow) mid-ventral to the neural tube. × 640

in only one 11-day old embryo. Although no accessory notochordal tissue could be identified, it did appear that a few extra-notochordal cells were present in the tail of this embryo. It should be noted that no abnormalities or duplications of the notochord were detected in the previous study of duplicitas posterior embryos (Center, 1969).

In comparison with duplicitas posterior embryos, all the abnormal Patch embryos showed varying degrees of compression or flattening of the notochord and varying amounts of cell-free space between the notochord and the neural tube (Fig. 7). However, all embryos, normal and abnormal, showed a well developed perinotochordal sheath. The sheath took up Alcian blue stain and showed as a well-defined, regularly thickened structure (Fig. 8). In the trunk region many sections of the abnormal embryos of the Patch stock showed normal development of the notochord in which no compression was detected. However, in other sections of the trunk level of these abnormal embryos a compressed notochord lost its medial position below the ventral surface of the neural tube and moved to the lateral margins of the neural tube. The thickness of the notochord did not remain constant throughout the serial sections in the abnormal embryos. The notochord appeared to be alternatively smaller or larger and irregular in shape.

We have assumed that the Patch embryos examined were homozygotes based on correlation with the morphological description of Patch embryos given by Grüneberg and Truslove (1960) in which only very minor abnormalities of the skull were found in the heterozygotes.

In certain anterior trunk sections of the Patch embryos, the notochord was very close to the neural tube, indeed almost touching it. In other anterior and posterior sections of the same embryos, the notochord was much farther away from the neural tube with a large region of cell-free space between the neural tube and

notochord (Fig. 9). Thus, a ventral shifting of the notochord was evident. The notochordal sheath in the abnormal embryos was found to be well-defined and regularly thickened but frequently undulated (Fig. 10). Moreover, the sheath was observed to be alternatively undulated or normal within a small number of successive sections (Figs. 11, 12). The Alcian blue staining of the perinotochordal sheath remained evident in all sections observed regardless of the change in shape of the notochord and the displacement of the notochord from its central position below the neural tube. Thus, it is clear that the integrity of the perinotochordal sheath was maintained throughout.

In striking contrast to the homozygous Patch (*Ph/Ph*) embryos the heterozygous Tail-short (*Ts*/+) embryos showed remarkable structural and morphological changes in the notochord and perinotochordal sheath. Varying degrees of disintegration of the notochordal sheath were observed. All the Tail-short embryos were from matings of the *Ts*/+ to +/+ mice, and thus, the abnormal ones were known to be heterozygotes.

Although in many sections of the abnormal Tail-short embryos, the notochord and the perinotochordal sheath appeared normal, other sections of the same embryos showed the eventual disintegration of the perinotochordal sheath. In the posterior trunk region of one 9 1/2-day (*Ts*/+) embryo, the perinotochordal sheath had undergone almost total disintegration in several sections. There was no well-defined notochord or sheath remaining. The Alcian blue staining appeared faint and ill-defined (Fig. 13), in comparison with sections of a normal embryo (Fig. 14). Anteriorly, in another 9 1/2 day (*Ts*/+) embryo, a clump of cells was all that remained of the notochord. Several sections showed that this clump of cells resembled the surrounding mesenchymal cells (Fig. 15). These cells were grouped into an abnormal notochord. Traces of the Alcian blue stain could still be observed indicating the presence of glycosaminoglycans which made up the perinotochordal sheath. The remnants of the sheath appeared thread-like and in the process of «breaking away» from the notochord. The cells that made up the former notochord did not always clump together, instead they separated and thus became indistinguishable from the rest of the surrounding mesenchymal cells; this was in marked contrast to the normal development in a 9 1/2 day embryo (Fig. 16). Lateral deflection of the *Ts*/+ notochord which was noted by Deol (1961), was confirmed in the present study.

Discussion

As noted above, it has been previously established that subdivision of the neural tube accompanied by duplication or reduction of hind-limb elements is a frequent component of the duplicitas posterior anomalies (Center, 1969). It is now evident that duplication of the notochord also occurs often in this syndrome although the duplicate notochordal mass is not easily recognized in some embryos, since it is seen in only a few serial sections and often is somewhat difficult to identify. The accessory notochord and sheath are not entirely normal in morphology. A similarity to the *Sd* mutant (Center et al., 1982) is the concentric swirling of mesenchymal cells observed in one 13-day duplicitas embryo. This abnormal arrangement of mesenchymal cells appears to be related to aberrant notochordal development and thus suggests the process is likewise anomalous in the duplicitas posterior embryo.

Since the notochord is deflected laterally and there is a misalignment of mesenchymal cells in some duplicitas embryos, it is possible that in cases where there is a second notochordal element the latter may be formed from the first by a bending process and separation of cells, perhaps due to loss of adhesion, resulting in a limited secondary axial element which is transitory.

Deficiencies in notochordal differentiation, including its sheath, have been interpreted as a controlling factor in the associated morphological abnormalities found in *Sd* mice (Grüneberg, 1958; Paavola et al., 1980; Center et al., 1982); it seems likely that notochordal and mesenchymal cell anomalies likewise play an important role in the duplicitas posterior morphological manifestations. It is noteworthy also that the intestinal and caudal vertebral anomalies often occur together in human abnormalities (Faris and Crowe, 1975), and it has been postulated that a «split» or doubling of the notochord is associated with these anomalies. Thus, the clustering of similar abnormalities in the duplicitas posterior syndrome is probably relevant in this regard, and the primary site of gene action, as has been advocated for *Sd* (Grüneberg, 1958; Paavola et al., 1980), may be the notochord, resulting in a secondary axis of development. Duplication of the neural tube has been reported in a number of mouse mutants, and the suggestion has been made that this diplomyelia results from the secondary formation of lumina and bifurcation of the neural tube. Notochordal anomalies are associated with some cases of diplomyelia (Cogliatti, 1986).

The present study revealed different types of abnormalities in the development of the notochord and the perinotochordal sheath in the Patch and Tail-short sections that were examined. The homozygous Patch had a normal basement membrane and normal development of the perinotochordal sheath, but abnormal orientation of an irregularly shaped notochord. In the Tail-short (*Ts*/+), the basement membrane and the perinotochordal tissue were found to be at various stages of disintegration, showing faint or no Alcian blue staining unlike the *Ph/Ph* mice. In the latter, there is strong Alcian blue staining of a well-defined and regularly thickened, but sometimes undulated sheath. Separation of the notochordal complex from the neural tube is a normal occurrence and this happens in the cranial-caudal direction. The anterior notochord complex in the *Ts* 10-day embryos examined generally separates from the neural tube even though the notochord may be irregular in shape or possess a poorly stained sheath.

The disorientation of the notochord in Patch mice can be explained by the presence of blebs. Blebs have been

observed in many abnormal mice also known as «hemorrhagic head» and «myelencephalic blebs» (Grüneberg, 1963). These blebs are initially filled with a clear liquid which subsequently becomes hemorrhagic. Certain defects of the eye and feet have been ascribed to these blebs. We have postulated that the movement of the notochord to different positions away from its normal, central position is due to some form of pressure being exerted on the notochord. This pressure appears to shift the notochord, but is not strong enough to destroy or damage the perinotochordal sheath. Apparently, the pressure, although not severe, is constant and continuous. The blebs, as mentioned, contain fluid initially and these have been observed extensively in the homozygous Patch (*Ph/Ph*) (Grüneberg and Truslove, 1960). Thus, blebs, are presumably responsible for the disorientation of the notochord and undulation of the sheath in the Patch embryos.

The results of the study of the Tail-short (*Ts*/+) demonstrate that notochord and sheath development are anomalous as early as the 9 1/2 day of gestation. The notochord and perinotochordal sheath fail to develop normally even though embryogenesis continues. The perinotochordal sheath stains poorly with Alcian blue indicating little or a negligible amount of glycosaminoglycans. This is found at most levels of the sections examined. In some Tail-short embryos the notochords were slightly deflected away from a medial position. This suggests that an abnormal association may exist between the neural tube and the notochord in the Tail-short mice. Our results thus indicate that abnormal development of the perinotochordal sheath and notochord may play an important role in the embryogenesis of *Ts* mice.

On the basis of differences in staining reactions as well as morphology, it is probable that the specific morphological target of gene action, directly or indirectly, is the notochord and perinotochordal sheath in all of the above studied anomalies. In the duplicitas posterior syndrome the primary effect of gene action appears to be the deflection or duplication of the notochord which is associated with incomplete development of the perinotochordal sheath. However, in Patch embryos, the effect on the notochord and perinotochordal sheath results secondarily from the formation of excess hydrops («blebs»). In contrast, a direct effect of the *Ts*/+ gene seems to be on the notochord and perinotochordal sheath. It appears that deflection of the notochord in all three instances plays a role in abnormal axial development; thus, normal development may be dependent on the presence of a notochord in a precise mid-ventral position below the neural tube. Recent work continues to support the importance of the notochord and perinotochordal materials in embryogenesis. McCaig (1986) has presented evidence showing that the notochord emits substances capable of influencing the directionality of myoblasts. Chondroitin sulphate proteoglycan in the notochordal extracellular material also may have a role in the migration of the neural crest and sclerotome cells (Newgreen et al., 1986). Although the abnormalities of the notochord and sheath are distinctive in duplicitas posterior, Patch and Tail-short mice, axial anomalies result in each case. Our observations thus establish the importance of the notochord and the perinotochordal sheath in the axial abnormalities found in the three mouse mutants. It is likely that the development of the notochord is influenced by the integrity of the basement membrane of the perinotochordal sheath. Hence, we plan to investigate the role of the components of the basement membrane, in particular, fibronectin and laminin in axial development.

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