In vitro chondrogenesis of limb mesoderm from normal and brachypod mouse embryos

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SUMMARY

This paper describes the cytodifferentiation of hind limb mesodermal cells from 12-day-old normal and brachypod (bpH) mouse embryos grown in vitro at high densities. Over a 3-day culture period normal cells underwent aggregation, nodule formation, and coalescence of nodules into large masses of cartilage. This was associated at the biochemical level with a cessation of cell division, with a concomitant increase in metachromatic matrix, and synthesis of collagen. Under the described culture conditions the collagen synthesized by 48 h cultures was predominantly of cartilage type with an α1:α2 ratio of 9:1. A change in the collagen synthetic program was observed when the entire medium was replaced after 48 h incubation with fresh, serum-free medium. Under these conditions the α1:α2 ratio was 4:1.

In contrast, brachypod cells plated at the same density appeared large, flattened, and stellate. Upon aggregation, normal nodule morphology was only rarely observed. More often large, irregular clusters formed from suspended cells loosely attaching to the surface aggregates. Concomitant with the marked changes in the morphology of the mutant cells was a linear increase in DNA synthesis and the appearance of many mitotic figures. A biochemical transformation in matrix synthesis was not observed, however. After a 24 h delay, mutant matrix accumulated and stained intensely with toluidine blue. Collagen was synthesized at approximately the normal rate and was of the cartilage type with an α1:α2 ratio of 9:1. When incubated in fresh, serum-free medium, the response of collagen subunit synthesis was identical to the normal cultures. In view of these results the possible manner in which brachypodism causes developmental anomalies of the limb skeleton is suggested.

INTRODUCTION

As a prelude to the formation of the skeletal limb elements, dense areas, commonly referred to as mesodermal condensations, appear in the limb mesoderm. It appears that these areas do not result from a local stimulation of cell division (Janners & Searls, 1970) but from a relative increase in cell packing density; whether this arises by aggregation towards the center of the condensations as suggested by Ede & Agerbak (1968) and as shown by Thorogood & Hinchliffe (1974) or by absence of centrifugal movement while cells outside of the condensations become more dispersed, as suggested by Gould, Day & Wolpert (1972), is not entirely clear. In either event, one can consider the

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mechanisms which regulate cell contact, cell movement, and cell density *in vitro* (Kohn & Fuchs, 1970) as possible *in vivo* control mechanisms involved in the formation of limb mesodermal condensations.

The importance of cell density in cartilage differentiation and pattern formation during limb development has been shown from studies on the *talpid* mutation in chickens (Ede, 1971). In their recent study on brachypodism, an inherited limb anomaly in the mouse, Grünberg & Lee (1973) proposed that the skeletal malformations could be ascribed to a reduction in the size of the mesodermal condensations. This finding was in agreement with earlier histochemical investigations of Milaire (1965), who indicated that a delay in matrix metachromacity in the brachypod limbs was related to an anomalous formation of the precartilaginous regions.

Over the past few years we have been investigating the abnormal development of the brachypod limb skeleton as a means of providing further insight into those processes which regulate normal limb development. In view of the above observations on the role of cell–cell interactions during early limb development as exemplified in both normal and mutant conditions, we decided to study the differentiation of 12-day-old normal and brachypod hind limb mesodermal cells cultured *in vitro* under conditions conducive to cartilage expression. This report describes some morphological and biochemical changes, which normal and mutant cells undergo during their cytodifferentiation into chondrocytes.

**MATERIALS AND METHODS**

Embryos were obtained from matings between parents of a Swiss albino mouse strain homozygous for the *bp* allele. The controls were from the same strain, but homozygous for the normal allele (+/+) at the brachypod locus. Staging of embryos was carried out as previously described (Krotoski & Elmer, 1973).

**Cell dissociation and culture conditions**

Hind limb-buds from 12-day-old embryos were washed with Ca²⁺- and Mg²⁺-free Tyrode’s solution (CMF) and left undisturbed for an additional 5 min. The CMF was removed and an enzyme solution (2-25 % trypsin–0-75 % pancreatin in CMF, pH 8-0) was added for 8-5 min. After enzyme treatment, the ectoderm was peeled away in Tyrode’s and the mesoblasts were collected in CMF solution. Approximately 30–40 min later the CMF solution was removed and the cells were dissociated in culture medium by gentle flushing with a capillary pipette. The culture medium consisted of Eagle’s BME containing 0-2 % l-glutamine (Grand Island Biological Co., Grand Island, N.Y.), with the addition of 50 units/ml of penicillin–streptomycin, and 10 % horse serum.

The cell concentration was determined by haemocytometer counting. Cell viability was tested by either trypan blue or erythrosine B exclusion. Viability was between 96–98 % for both brachypod and normal cells. For culturing, cells
were plated at a density of 4500 cells/mm² in a 30 ml plastic T-flask (Falcon Plastics) containing 4 ml of media. This concentration has been shown to be conducive to cartilage formation by mouse limb-bud mesenchyme \textit{in vitro} (Umansky, 1966). The flasks were gassed with a 5% CO₂-95% air mixture at a pH of 7-0 and incubated at 37 °C. The medium was renewed after 48 h by removing 2 ml and replacing it with 2 ml of fresh medium.

\textit{Histochemical and biochemical analyses}

Cells were fixed with 10% buffered formalin. Toluidine blue or alcian blue were used to stain for mucopolysaccharides. Other cells were stained with haematoxylin and counterstained with either eosin or orange G.

DNA analysis was carried out by homogenizing cells at 2 °C in a 0·1 M Tris-HCl buffer, pH 7-2, containing 0·1% sodium dodecyl sulfate. The homogenate was precipitated upon addition of an equal volume of cold 20% trichloroacetic acid (TCA) and the DNA was extracted from the precipitate with 5% perchloric acid at 90 °C. DNA assays were carried out by the method of Burton (1956) as modified by Giles & Myers (1965).

The amount of polypeptide-bound hydroxyproline in the cells plus the medium over the 3-day culture period was used as a measurement of collagen synthesis. Medium which had not been used for culturing provided the blank. The medium and cells were collected and exhaustively dialyzed against running tap water. After dialysis the samples were lyophilized to dryness, hydrolyzed in 6 N-HCl, and assayed for hydroxyproline by the method of Switzer & Summer (1971). The Student \( t \) test was used to determine significance of the differences.

To determine the type of collagen being synthesized, cells were incubated for 2 days under normal culture conditions. In one series of experiments, half of the medium was removed from the cultures and replaced with fresh medium plus 2 \( \mu \)Ci/ml of L-[U-\( ^{14} \)C]proline (sp. act. 255 mCi/mmole, New England Nuclear Corp.), 100 \( \mu \)g/ml of \( \beta \)-aminoproprionitrile (to inhibit cross-linking in collagen), and 50 \( \mu \)g of ascorbic acid. The cultures were incubated for an additional 24 h.

Collagen was extracted from the medium at 4 °C with 1·0 M-NaCl in a 0·05 M Tris-HCl buffer, pH 7·5, as described by Miller & Matukas (1969). Skin collagen (15 mg) from lathyritic rats was added to the extract to aid in the precipitation of the radioactive collagen during dialysis against 0·05 N-Na₂HPO₄. The precipitates were collected, purified twice by dissolving in 0·5 M acetic acid and precipitation with 5% NaCl (w/v), before dialysis against starting buffer (0·04 M sodium acetate, 1 M urea, pH 4·8) for chromatography on a carboxymethyl cellulose (CMC) column. To ensure complete denaturation of the triple helix, the collagen solutions were warmed to 43 °C for 30 min before applying them to a 1·5 x 100 cm column of CMC (Whatman, microgranular CM-32, capacity 1·0 m-equiv/g). The column was maintained at a temperature of 42 °C.
and the collagen subunits were eluted with a linear salt gradient (0·0–0·1 M-NaCl). Five ml fractions were collected and 0·5 ml aliquots from each fraction were counted with 10 ml of a scintillation fluid consisting of 0·4 % (w/v) 'Omnifluor' (New England Nuclear Corp.) in toluene, and 10 % (v/v) of Beckman 'Biosolv BSS-3'.

In another series of experiments the entire medium was replaced after 48 h of incubation with fresh medium as described in the preceding experiment except that it was serum-free and the radio-isotope used to label the collagen in the normal cultures was 5 μCi/ml of [14C]glycine (sp. act. 102 mCi/m mole, New England Nuclear Corp.) and in the mutant cultures it was 10 μCi/ml of L-[5-3H]proline (sp. act. 20 Ci/m mole, Schwartz-Mann). The cultures were incubated for an additional 16 h.

In these experiments the normal and brachypod media were pooled together, 15 mg of rat skin collagen was added, and the medium was then treated with pepsin as described by Layman, McGoodwin & Martin (1971). After inactivation of the pepsin, the medium was dialyzed against starting buffer used for the CMC column and the collagen subunits were separated and assayed for radio-activity as previously described.

RESULTS

Cytodifferentiation of normal and brachypod hind limb mesoderm in vitro

Normal cultures

In 12-day-old mouse embryos the mesodermal condensations in the hind limbs are apparent; however, histological differences between the cells are not yet recognizable (Milaire, 1963). Freshly dissociated cells were spherical and of equal size. They contained large nuclei, prominent nucleoli, and a relatively small amount of cytoplasm.

When placed into culture the cells began to take on a variety of shapes within the first 6 h. A few remained rounded, some developed long, tapering, cytoplasmic projections, while many became tear-shaped (Fig. 1). As incubation continued the cells moved along the surface and upon coming into contact with each other in areas of high density, tended to aggregate into compact, multicellular clusters. By 24 h many of these clusters assumed a rounded shape and were comprised of cells actively secreting metachromatic matrix material (Fig. 2).

Within the next 24 h the clusters grew considerably in size with the cells

Figs. 1–3. Phase-contrast micrographs of nodule formation in aggregating 12-day normal mouse hind limb-bud mesodermal cells after 6, 24, and 48 h of culture. A few spindle-shaped cells (arrow) could be seen overgrowing the monolayer sheets by 48 h of incubation. × 163.

Fig. 4. Light micrograph of a normal 72 h culture in which nodules have coalesced to form a ridge of cartilage with a large conglomerate projecting from it. × 20.
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piling upon each other to form small mounds or nodules of cartilage (Fig. 3). These nodules were of approximately 0.5 mm in diameter. They contained large, rounded masses of cartilage-secreting cells bordered by a double layer of oblong cells reminiscent of a perichondrium. Overlying the cells surrounding the nodules were a few spindle-shaped cells which may have been destined to form muscle cells. Incubations were never long enough to determine the extent of myogenesis in these cultures.

By 72 h of culture the nodules increased considerably in number and in many cases fused together to form ridges of cartilage with large amorphous masses projecting from them (Fig. 4). With only slight movement of the culture flask, the multilayered conglomerate of cartilage often detached from the ridge and floated free in the medium.

**Brachypod cultures**

In 12-day-old brachypod mouse embryos there is no apparent morphological difference in the hind limbs from their normal counterparts (Milaire, 1965; Grüneberg & Lee, 1973). Histologically the mesoderm cells also have a normal morphology. When placed into culture, however, they deviated strikingly from the shapes assumed by the normal cells. First of all those which attached to the surface of the culture flask tended to flatten out rather quickly. Many, however, did not attach and remained floating in the culture medium. In areas of low density the cells were large, flattened, and stellate with conspicuous pseudopodia (Fig. 5). In fact these cells had a morphological appearance much like chondroblasts that have been cultured in the presence of 5-bromo-2'-deoxyuridine (Holtzer & Abbott, 1968; Schiltz, Mayne & Holtzer, 1973). As they moved toward centers of aggregation where there was increasing cell contact, they seemed to become 'fibroblastic-like' before rounding up as they became positioned within the aggregate. The 24 h aggregates did not stain positive with either toluidine blue or alcian blue.

During the next 48 h the cell aggregates increased in size and on rare occasions would assume the typical normal nodule morphology. However, the most predominant observation was the formation of large, irregular clusters of cells (Figs. 6, 7). These clusters appeared to form from the loose attachment between the cells suspended in the culture medium and the surface aggregates. By the second day of incubation staining with toluidine blue and alcian blue was as
intense as the normal cultures. Although ridges of cartilage could be seen in 3-day cultures, the large amorphous masses projecting from them were not observed.

In some areas of the culture flask the cells would form compact monolayer sheets with many spindle-shaped cells overlying them (Fig. 8). The cells within the sheets would often show mitotic figures. In normal cultures the cells in dense monolayers contained comparatively fewer mitotic figures.

**Biochemical analyses**

Morphological changes in the cell surface have been correlated with changes in nucleic acid and protein biosynthesis (Todaro, Lazar & Green, 1965; Pardee, 1971). To determine whether these processes were also being regulated in the brachypod cultures, measurements of DNA content over time were made as an indicator of cell proliferation and quantitative and qualitative assays of collagen were carried out since it comprises the major protein component of the cartilage matrix. The qualitative assay of collagen provided information concerning the question of whether the cytological changes in the brachypod cultures could be correlated with a biochemical transformation in the subunit composition of the collagen being synthesized (Layman, Sokoloff & Miller, 1972; Schiltz et al. 1973). Collagen of the type \([\alpha 1(II)]_3\) has been shown to be specified by the chondrocyte genome (Miller & Matukas, 1969; Trelstad, Kang, Igarashi & Gross, 1970), whereas, fibroblastic type is designated as \([\alpha 1(I)]_2\alpha 2\) (Piez, Eigner & Lewis, 1963).

Fig. 9 shows the DNA content of normal cultures increased by 50% during the first 24 h of incubation. Between 24–72 h no further increase in DNA content was observed (Fig. 9). This seems to reflect the cytological observations which showed during this same period an increase in nodule formation surrounded by large areas of dense sheets of cells (see Fig. 3). Only rarely were mitotic figures observed in these regions.
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In brachypod cultures the DNA content increased linearly over the 3-day period (Fig. 9). This supports the observation of mitotic figures in both areas of high and low density during the entire culture period. Both attached and floating cells were collected for DNA analyses. If the attached cells only represented the population of surviving cells, a linear increase in DNA content should not have been observed (Caplan & Stoolmiller, 1973). It is not understood why many of the mutant cells did not attach to the surface of the culture flask, but it is known from autoradiographic experiments that these 'floaters' can incorporate [³H]thymidine into their DNA (Elmer, unpublished observations).

In the experiments carried out to determine the amount of collagen synthesized with increasing time of incubation, values were corrected for a small amount of non-dialyzable hydroxyproline that was present in the horse serum. Freshly dissociated limb mesodermal cells from normal and brachypod 12-day-old embryos contained a small, but similar amount of collagen (Fig. 10). Both cell types also synthesized the same amount of collagen during the first 24 h of culture. Between the first and third days the production of collagen was slightly greater in the normal cultures (Fig. 10). The difference examined by the t-test was found to be insignificant (\( P > 0.2 \)). The observations indicate, therefore, that (1) brachypodism has no dramatic effect on the relative amount of collagen synthesized by limb mesodermal cells grown \textit{in vitro}, and (2) the amount of collagen synthesized does not seem to play a major role in the histotypical expression of the cells. The latter finding agrees with the studies of Schiltz \textit{et al.} (1973) on chondroblast transformation \textit{in vitro}.  

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**Fig. 10.** Collagen content in cells plus medium from 12-day normal and brachypod embryonic hind limb-bud mesodermal cultures. ○—○, Normal; ⋅—⋅, brachypod.
Although the quantitative data on collagen synthesis is useful, information on the type of collagen being synthesized is required to determine the nature of the cells that we are dealing with (Levitt & Dorfman, 1974). We characterized the composition of the collagen subunits synthesized by the cells under two very different sets of conditions. In one series of experiments we replaced half of the culture medium (2 ml) with fresh medium plus $[^{14}\text{C}]$proline, ascorbic acid and $\beta$-aminoproprionitrile. In another series of experiments the entire culture medium (4 ml) was replaced with the above medium except that it was serum-free and contained $[^{14}\text{C}]$glycine in the normal cultures and $[^{5}\text{H}]$proline in the brachypod cultures. By using the serum-free medium and the double label we were able to isolate and characterize the collagen subunits from both cultures under identical conditions without going through the salt extraction. Furthermore, the two experiments allowed us to determine whether the normal and brachypod cells would respond similarly to a modulating influence the culture medium may have on the type of collagen subunits being synthesized.

From the elution profile of the radioactive collagen synthesized by the cells in cultures containing serum, it is clear that both the normal and mutant cells synthesized predominantly $\alpha_1$ type collagen (Fig. 11). The difference in the elution position of the radioactive $\alpha_1$ chains from the rat skin carrier $\alpha_1$ chains
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Fig. 12. Carboxymethylcellulose chromatogram of collagen subunits synthesized by normal and brachypod 12-day embryonic hind limb-bud mesodermal cultures. After 48 h of incubation, normal cultures were labeled with [14C]glycine and brachypod cultures with L-[5-3H]proline for 16 h in fresh, serum-free medium. Rat skin collagen, optical density; O—O, normal cultures, radioactivity; •—•, brachypod, radioactivity.

can be attributed to their amino acid composition. A small amount of radioactivity was also observed in the α2 region which suggests the presence of some fibroblast cells in the cultures (Fig. 11). However, the α1/α2 ratio was 9:1, a value more characteristic of cartilage collagen. These data show, therefore, that even though the brachypod cells are not histotypically comparable to their normal counterpart, they can on the basis of the type of collagen synthesized still be called chondrocytes.

In the second series of experiments an increase in the radioactivity of the α2 peak was observed (Fig. 12). This indicates that the change in the culture conditions was sufficient to cause a modulation in the type of collagen being synthesized. An α1/α2 ratio of 4:1 was obtained within the short labeling period (16 h). This suggests that these cells have not become very stabilized within the 48 h prior to the change in the culture media. Taken together then the two experiments indicate that even though normal and brachypod cells show a morphologically differential response to growth in vitro, they both respond similarly at the transcriptional and translational levels with respect to collagen synthesis.
DISCUSSION

Isolated hind limb mesodermal cells from normal and brachypod embryos are morphologically indistinguishable. However, in situ studies have revealed that before the onset of chondrogenesis a spatial heterogeneity in the expression of the brachypod gene can be detected in the organization of the limb mesoderm. The blastemata of the most severely affected limb elements have been found to be reduced in size and delayed in their production of a metachromatic matrix (Milaire, 1965; Grüneberg & Lee, 1973). The use of the entire limb mesoderm notwithstanding, the present studies showed that the determinants of the brachypod effect in situ can also act on the sequence of events required for the morphological transition of mesodermal cells to chondrocytes in vitro. This system provides us, therefore, with a means of probing into the ultimate nature of the disturbances brought about by the brachypodism gene.

The typical sequence of events at both the morphological and biochemical levels that has been described by other investigators in the cytodifferentiation of mesodermal cells into chondrocytes was also observed in our normal cultures (Moscona & Moscona, 1952; Umansky, 1966; Caplan, 1970). At the cellular level there was an initial aggregation into compact clusters which soon differentiated into cartilage nodules followed by a fusion of these nodules into ridges and large conglomerates of cartilage. It should be stressed that clusters did not develop merely as a consequence of overcrowding since many were seen in areas which were not covered entirely with cells. The biochemical events which paralleled the morphological changes were a cessation of cell division with a concomitant increase in mucopolysaccharide staining, and the specific synthesis of cartilage-type collagen.

In contrast, the brachypod cells demonstrated deviation from culture inception. They immediately flattened, forming large stellate cells with a large number remaining suspended in the media. Many of these ‘floaters’ attached loosely to the aggregates of cells on the surface of the flask giving the appearance of nodule formation. These nodules did not illustrate the normal morphology, being composed of large-sized and irregularly shaped clusters of cells. Furthermore, even though ridges of cartilage could be observed, the large amorphous masses were not observed.

In many areas of the brachypod cultures, dense monolayers composed of epithelioid cells could be seen containing many cells in mitosis. The appearance of nodules was noticeably absent. In addition, a metachromatic matrix was only faintly apparent. Similar results have been obtained when monodispersed cultures of chondroblasts were grown on a plasma clot (Holtzer, Abbott, Lash & Holtzer, 1960; Abbott & Holtzer, 1966) or in the presence of histotypically different cells (Abbott & Holtzer, 1964). From data such as these, the suggestion has been made that the initial steps in transforming the cell’s synthetic activities involves an alteration of the cell membrane (Holtzer & Abbott, 1968).
Brachypodism effect on chondrogenesis

Recently Schiltz et al. (1973) showed that environmentally induced changes in the morphology of cultured chondroblasts can be correlated with a biochemical transformation in the type of matrix components synthesized. Normally chondroblasts synthesize chondroitin sulfate A, C, and \([\alpha 1(II)]_3\) type collagen. When cultured in BUDR (5-bromodeoxyuridine) or embryo extract they synthesize predominantly hyaluronic acid and \([\alpha 1(I)]_2\alpha 2\) type collagen, suggesting a transformation into fibroblasts. In the brachypod cultures, however, the situation does not seem to be as clearly defined. With respect to collagen synthesis a concomitant transformation at the biochemical level does not take place. Actively dividing brachypod cells were still able to synthesize predominantly \([\alpha 1(II)]_3\)-type collagen.

Because analyses of mucopolysaccharide accumulation was followed by metachromatic staining, conclusions concerning a possible transformation in the type being synthesized cannot be reached. The histochemical analyses did demonstrate a 24 h delay in metachromatic staining, a finding similar to what occurs in situ prior to cartilage formation (Milaire, 1965). After this delay, the staining intensity in the \(bp^H\) cultures was as great as in the normal cultures. In membranous bone development a high chondroitin sulfate to collagen ratio has been shown to favor chondrogenesis (Hall, 1970). Therefore, instead of a qualitative difference in mucopolysaccharide synthesis, it may have been quantitatively reduced enough to seriously affect chondrogenesis in the brachypod cultures. This possibility is presently being investigated.

A number of environmental factors have been shown to affect chondrogenic expression in vitro (Levitt & Dorfman, 1974). One example has been the demonstration of conditioned-medium factors in both limb-bud mesodermal cultures (Schacter, 1970\(a, b\)) and embryonic chondrocyte cultures (Solurish & Meier, 1973). These factors have been reported to influence the synthesis of both chondroitin sulfate and collagen. Solurish, Meier & Vaerewyck (1973), proposed that the modulation in the production of the extracellular matrix results from a positive feedback mechanism on the synthetic machinery of the chondrogenic cell.

There is evidence of two different hereditary skeletal mutants which produce factors that influence the growth of embryonic long bones in vitro. One is the "Creeper" mutation in chickens (Elmer, 1968) and the other is brachypodism (Konyukhov & Bugrilova, 1968). It has been reported that extract from 13-day-old embryos homozygous for \(bp^H\) significantly reduced the growth of normal 13-day limb elements in organ culture (Ginter & Konyukhov, 1966). Evidence that the synthesis of the growth inhibitor was under the control of the mutant gene was produced when extract from 13-day-old embryos heterozygous for the \(bp^H\) allele suppressed the growth in vitro of normal tibiae by approximately half as much as extract from homozygotes (Bugrilova & Konyukhov, 1971). Recently it was reported that this factor has been purified 400-fold and preliminary characterization suggests it is a protein with a
molecular weight of 76000 (Pleskova, Rodionov, Bugrilova & Konyukhov, 1974). It has been proposed that the brachypodism gene is exerting its effect on the cytodifferentiation of limb mesodermal cells through the production of this extracellular protein.

With the available information it seems possible to come to some general conclusions about the manner in which brachypodism is causing disturbances in the development of the limb skeleton. First, the mutant gene is acting on processes independent of those which control the synthesis of the specific types of collagen and possibly mucopolysaccharides characteristic of the cartilage phenotype. This is supported by the facts that (1) limb mesodermal cells grown in vitro synthesize \([\alpha 1(II)]_3\) type collagen and after a short delay stain intensely with toluidine blue, (2) normal and mutant mesodermal cells show the same identical shift in their genetic programming for collagen synthesis when grown in fresh, serum-free medium, and (3) the \(bp^H\) neonatal fibula, which is one of the more histologically abnormal limb elements, synthesizes \([\alpha 1(II)]_3\) collagen and stains metachromatically with toluidine blue (Rhodes & Elmer, 1973).

Secondly, brachypodism is acting at the site of the cell membrane which is causing an interference with cell functions peculiar to the developmental program of the skeletal limb elements. Support for this contention are the observations that (1) brachypodism disturbs only appendicular skeletal development (Landauer, 1952), (2) the anomalies of the limb skeleton are related to a possible misallocation of material between blastemata, suggesting a disturbance in cell adhesion (Grüneberg & Lee, 1973), (3) the formation of large, flattened, stellate cells in vitro reflect a change in the cell membrane, (4) in culture \(bp^H\) cells loosely attach to each other as well as the substrate, and (5) there is a continued synthesis of DNA and the appearance of mitotic figures in vitro which in other culture systems has been correlated with membrane disturbances (Todaro, Lazar & Green, 1965; Castor, 1969).

Thirdly, brachypodism is mediated through the production of a growth factor. Support for this hypothesis comes from the investigations of Konyukhov and his collaborators who have presented strong evidence that the \(bp^H\) gene directs the synthesis of a growth-inhibitory factor. In addition, we have preliminary evidence which suggests that mutant cells release a factor into the culture medium which can reduce nodule formation in normal cultures. We are presently determining whether this factor is analogous to the protein component prepared by Pleskova et al. (1974) from 13-day brachypod embryo extract.

A preliminary report of the findings in this paper was presented at the American Zoologist Meeting, Houston, Texas, December 1973.
REFERENCES


(Received 4 June 1974)