Analysis of glycosaminoglycans during chondrogenesis of normal and brachypod mouse limb mesenchyme

By JANET SHAMBAUGH 1 AND WILLIAM A. ELMER 2

From the Biology Department, Emory University, Atlanta, Georgia

SUMMARY

Studies have been carried out to characterize radioactive incorporation rates and steady-state levels of hyaluronic acid (HA) and chondroitin sulfate (CS) as well as hyaluronidase activity in the hind limbs of normal and brachypod mouse embryos between 11–14 days of gestation. The results of the analysis show that changes in the synthetic and degradative rates of HA and CS occur at about the 12.5-day stage in normal hind limbs. These changes include an increased rate of CS synthesis, a decreased rate of HA synthesis, and a correspondingly sharp, transitory rise in hyaluronidase activity. Similar changes also occur in brachypod hind limbs but appear to be delayed in onset by approximately one-half day. In addition, the mutant hind limb exhibits a slower loss of HA at a time when turnover would be expected to be on the increase. These changes are concomitant with cell surface alterations and abnormal mesenchymal condensation formation which have been previously shown to occur in this mutant.

INTRODUCTION

Changing physiological levels of the cartilage glycosaminoglycans (GAGs), hyaluronic acid (HA) and chondroitin sulfate (CS), are thought to be important to chondrogenesis, and therefore, to normal skeleton formation. Thus, a correlation has been shown between HA and morphogenesis in several developing systems (Polansky, Toole & Gross, 1973; Toole, 1973a; Pratt, Larson & Johnston, 1975; Solursh, 1976; Greenberg & Pratt, 1977; Solursh & Morriss, 1977; Derby, 1978, Orkin & Toole, 1978), and removal of HA has been correlated with subsequent cytodifferentiation of chondrocytes (Toole, 1972; Corsin, 1975; Smith, Toole & Gross, 1975). While most studies of chondrogenesis have focused on CS or CS proteoglycan in the chick limb system (Goetinck, Pennypacker & Royal, 1974; Hascall, Oegema, Brown & Caplan, 1976; DeLuca et al. 1977; Holtzer, Okayama, Biehl & Holtzer, 1978) and in the mouse limb system (Royal & Goetinck, 1977), changing levels of both GAGs have been described using only radioactive labeling in chick limb development (Toole, 1972) and newt limb regeneration (Toole & Gross, 1971; Smith et al. 1975). A few studies have measured actual levels of HA and CS – one in chick axial skeleton (Kvist & Finnegan, 1970) and another in neurulating chick (Derby, 1978).

1 Author’s address: Biology Department, Johns Hopkins University, Baltimore, Maryland 21218 U.S.A.
2 Author’s address: Biology Department, Emory University, Atlanta, Georgia 30322, U.S.A.
The present study characterizes radioactive incorporation rates and steady-state levels of HA and CS, as well as hyaluronidase (HAdase) activity, during the early stages of hind-limb development in normal (+/+) mice and mice which carry the brachypod (bp^H/bp^H) mutation. This mutation, which affects the differentiation and development of the appendicular skeleton, appears to act by causing a delay and reduction in the formation of the mesenchymal condensations which subsequently develop into the cartilaginous anlagen of the skeletal elements (Grüneberg & Lee, 1973; Elmer, 1976). The defect in the condensation process has been correlated with alterations in the surface properties of the prechondrogenic mesenchyme (Duke & Elmer, 1977, 1978, 1979; Hewitt & Elmer, 1978). It has not been reported, however, whether biochemical differences of extracellular matrix components are being displayed concomitantly during this developmental time period. The data presented in this paper indicate that an alteration in the levels of the glycosaminoglycans is also occurring at this time period.

MATERIALS AND METHODS

Source of tissue

Normal and mutant mice were obtained from Jackson Laboratory, Bar Harbor, Maine. Homozygous bp^H/bp^H mice were bred onto the same hybrid background as the phenotypically normal mice (CAF_1/J). Embryos of a particular gestational age were generated using timed matings with the appearance of the vaginal plug called day zero. Embryos were removed from the uterus in warm, sterile Tyrode's solution and staged as described previously (Elmer & Selleck, 1975). The hind-limb buds were excised using iridectomy scissors and either placed into organ culture (Kochhar & Aydelotte, 1974) or treated further for cell culture (Elmer & Selleck, 1975). For culturing the whole limb bud, the medium consisted of 75% BGJ chemically defined medium (Difco Laboratories), 25% fetal calf serum (GIBCO), 150 μg/ml ascorbate (Nutritional Biochemicals Corp.), and 50 μg/ml gentamicin sulfate (Garamycin, Schering Pharmaceutical Corp.). For cell culture, limb mesenchyme was obtained by removing the ectoderm with microneedles after a 5 min incubation at room temperature in 2-25% trypsin (GIBCO) plus 0-75% pancreatin. The resulting mesodermal core was placed in calcium-magnesium-free Tyrode's solution for 30 min at 25 °C to dissociate the cells. The cells were plated in 35 mm plastic tissue culture dishes (Falcon) at a density of 1 × 10^6 cells/ml in 3 ml Eagle's Basal Medium with Hank's salts supplemented with glutamine (GIBCO), 10% horse serum (GIBCO), and 50 μg/ml gentamicin sulfate. Both cell and organ cultures were incubated in a humidified atmosphere of 5% CO_2, 95% air, at 37 °C.

Quantification of glycosaminoglycans

The radioactive precursor D-[6-3H]glucosamine-HCl (sp. act. 20–30 Ci/mmol, Amersham–Searle) used in the synthesis of HA, CS and glycoproteins
Glycosaminoglycan levels in normal and brachypod limbs

(GPs) was added at \( t_0 \) to the medium at a concentration of 5 \( \mu \text{Ci/ml} \) for the organ cultures and 1 \( \mu \text{Ci/ml} \) for the cell cultures. Since the incorporation rate was observed to be linear for at least 12 h for both genotypes, a 6 h labeling period was used throughout all of the experiments. Cell and organ cultures were also labeled concurrently with 5 \( \mu \text{Ci/ml} \) of Na\(^{35}\)SO\(_4\) (carrier free, Amersham-Searle) as an additional indicator of sulfated GAGs. The GAGs were extracted from either one cell-culture plate (cell layer plus medium) or from 10 limb buds combined with 20 unlabeled limb buds by shaking in 0.5 M-NaOH and 1.0 M sodium borohydride overnight at 25 °C. After dialysis against running water overnight, an aliquot was removed for DNA determination (Giles & Myers, 1965). For the limb-bud retentate, the GAGs were precipitated with three volumes of cold ethanol plus 5% potassium acetate, and chromatographed on a column of DEAE-52 cellulose (Whatman) with a linear gradient of 0-1-2 M-LiCl in 0.05 M tris buffer, pH 7.2 (Pratt, Goggins, Wilk & King, 1973). Fractions were collected (3 ml) and a 0.5 ml aliquot of each fraction was assayed for radioactivity in 10 ml of ACS (Amersham-Searle) liquid scintillant using a Beckman LS 100 counter to determine the elution profile. The fractions within each peak (fractions 14-20 for HA and 22-37 for CS) were pooled, dialyzed, concentrated to 1.0 ml using a Molecular Separator Kit (Millipore) and assayed for uronic acid content (Bitter & Muir, 1962) and radioactivity. The identity of the peaks was confirmed by digesting the concentrated effluent with enzymes of narrow specificity: 1.0 mg/ml leech head hyaluronidase (Biotrics, Inc., Arlington, Mass.), 5 units/ml chondroitinase AC or ABC (Sigma) in enriched tris buffer (Saito, Yamagata & Suzuki, 1968), and 1.0 mg/ml heparanase (kindly supplied by A. Linker, V. A. Hospital, Salt Lake City, Utah) in 0.1 M sodium acetate buffer, pH 7.0 (Linker & Hovingh, 1972).

HA levels in limb-bud extracts were also quantified using the Reissig test (Reissig, Strominger & Leloir, 1955) after digestion with *Streptomyces* HA\(_\text{Dase}\) (Calbiochem, Grade B) as described by Hatae & Makita (1975). For this assay the NaOH extract was first deproteinated by precipitation with 10% trichloroacetic acid (TCA). The TCA was extracted with three changes of an equal volume of ethyl ether, and the GAGs were precipitated from the aqueous phase with 3 vol. of cold ethanol containing 5% potassium acetate. The GAGs were dissolved in 0.2 ml of 0.05 M sodium acetate buffer, pH 5.0, and two Turbidity Reducing Units (TRU) of Streptomyces HA\(_\text{Dase}\) were added. After incubation overnight at 55 °C, the pH was adjusted to 9.2 before measuring free hexosamine reducing ends.

Cell cultures were extracted in a similar manner. As described above, excess protein was first removed from the dialysis retentate using TCA. Separation of GAGs was then accomplished by sequential collection on a filter of pore size 0.45 \( \mu \text{m} \) (Millipore) according to methods of Saarni & Tammi (1977). The scintillant used was ACS (Amersham-Searle).
Loss of glycosaminoglycans in culture

Approximation of GAG turnover in limb-bud organ cultures was measured by pulse labelling for 6 h followed by a 4-day incubation in medium minus the radioactive precursor and containing a 50-fold excess (10 \( \mu \text{M} \)) of unlabeled glucosamine. Cultures were fed by changing 1 ml of medium each day. Cultures were terminated at 1-day intervals by extracting four limb buds as described above. The extract was dialyzed, and DNA content and radioactivity determined.

HAdase activity in limb-bud homogenates

HAdase activity was measured in a crude homogenate of 50–100 limb buds by the Reissig test (Reissig et al. 1955) using 100 \( \mu \text{g} \) of HA as substrate (Sigma, Umbilical Cord) for a 20 h incubation at 37 °C (Polansky, Toole & Gross, 1973). Alternatively, \(^{3}\text{H}\)HA isolated from limb-bud cultures by DEAE chromatography was used as substrate. A unit of activity is defined as \( \mu \text{g} \) HA digested/10 limb buds/24 h.

RESULTS

Glycosaminoglycans were quantified over developmental time in two ways in order to obtain a more complete description of the changes occurring in the limb bud. In one assay, a colorimetric analysis of the GAG levels was carried out to determine the quantity of GAGs at different stages of early development. The other assay involved the use of the radioactive precursors, \(^{3}\text{H}\)glucosamine and Na\(^{35}\text{SO}_4\), to determine the amount of HA, CS and GPs produced during a particular 6 h period. However, since these values are determined by synthesis minus degradation, the loss of radioactivity was also measured to determine the importance of degradation to rate. The data are expressed on either a per limb bud or per \( \mu \text{g} \) of DNA basis since the DNA content was not significantly different between the two genotypes in limb buds of the same age (Table 1).

To perform the colorimetric and radioactive assays, HA, CS and GP were
Glycosaminoglycan levels in normal and brachypod limbs 229

first separated by anion exchange chromatography as described under Methods. A sample chromatograph is shown in Fig. 1, indicating how the fractions were pooled to give three groups. Peak one co-elutes with HA standard, whereas, peak two which contains both 3H and 35S co-elutes with CS standard. The exact nature of the components present in the two major peaks was confirmed using enzymes with narrow specificities. These results are summarized in Table 2. The first major peak is 90% digestible by leech hyaluronidase. This enzyme has absolute specificity for HA. The second, broader peak is about 40% chondroitin sulfate A and C, 30% chondroitin sulfate B, and 20% heparan sulfate. The remaining 10% could be keratin sulfate since this GAG is not degraded by any of the enzymes used. Since the first major peak contains mainly HA and the second peak contains mainly CS, HA and CS were routinely quantified by summing the fractions (14–20 for HA and 22–37 for CS) within their respective peaks. All fractions collected before the HA peak were also pooled to quantitate the GPs (Pratt et al. 1973). The percent composition of the peaks did not differ significantly between the two genotypes (Table 2), therefore HA and CS may be compared between genotypes by measuring the material collected within the two peaks.

Table 2. Identification of components in the DEAE column effluent using specific enzyme digestions

<table>
<thead>
<tr>
<th>Peak 1*</th>
<th>Day 11-5</th>
<th>Day 12-5</th>
<th>Day 13-5</th>
<th>Day 14-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>% digested by leech HAdase</td>
<td>72 (74)†</td>
<td>88 (92)</td>
<td>90</td>
<td>94 (86)</td>
</tr>
<tr>
<td>Peak 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% digested by CSase ABC</td>
<td>58 (49)</td>
<td>65 (68)</td>
<td>75</td>
<td>72 (61)</td>
</tr>
<tr>
<td>CSase AC</td>
<td>—</td>
<td>35 (35)</td>
<td>40</td>
<td>—</td>
</tr>
<tr>
<td>Heparanase</td>
<td>—</td>
<td>17 (16)</td>
<td>19</td>
<td>—</td>
</tr>
<tr>
<td>Leech HAdase</td>
<td>0 (0)</td>
<td>—</td>
<td>—</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

* Peak 1 (fractions 14–20) represents HA, peak 2 (fractions 22–37) is CS.
† Values in parentheses are results from digestion of pb^+/bp^+. All other values are from +/+ limb mesenchyme extracts.

Chondroitin sulfate measurements

The radioactive incorporation of [3H]glucosamine into CS (Fig. 2A) displays a constant rate until 12-5 days of gestation, when the rate increased linearly (r² = 0.97 for linear regression of line described by last four points, n = 2). The timing of this abrupt change corresponds to the onset of visible areas of cell condensation and cartilage matrix deposition in the normal limb. In the mutant, condensations are also visible, however, they are smaller in size (Grüneberg & Lee, 1973) and contain a lesser amount of matrix material (Duke & Elmer,
Fig. 1. A representative DEAE-cellulose chromatogram of glycosaminoglycans from 13-day +/+ mouse hind limbs. \([3H]\)Glucosamine and \(\text{Na}_2\text{SO}_4\), each at 5 \(\mu\text{Ci/ml}\), were added at \(t_0\) to the organ culture medium for a period of 6 h. Ten limb buds were extracted together with 20 unlabeled buds, and the GAGs precipitated with ethanol before being applied to the column. Fractions were tested for radioactivity, and the material within each peak was pooled as indicated by the vertical lines. The concentrated peaks were tested for uronic acid content and enzyme susceptibility. Peak one represents HA and peak two is CS. Solid line, \([H]\) counts; broken line, \([35S]\) counts.

Although the synthetic rates coincide closely, the change at 12.5 days does not appear to be quite as sharp in the mutant limb bud as compared to the normal control.

The increase in micrograms of CS over developmental time is shown in Fig. 2D. The quantitative levels in the limb buds at the different ages are consistent with the integral of the rate function, as expected. In other words, the linear increase in micrograms (Fig. 2D) corresponds to the constant segment in the rate (Fig. 2A) while a change to quadratic increments (Fig. 2D) after 12.5 days of development corresponds to the linear portion of the rate function in Fig. 2A.* When this regression line is used to calculate \(x\) values (time in days) for the actual \(y\) values for +/+ (micrograms of CS), the accumulation of CS in the brachypod limbs is observed to lag significantly \((P < 0.01)\) behind their normal counterparts for each point past day 12 (paired \(t\) test for last four time points in Fig. 2D). The lag amounts to nearly one-half day by day 12.5 through 14.5 (mean \(\pm\) S.D. = 10 h \(\pm\) 0.5 h).

* A regression line can be fitted to these data by weighting the time points that correspond to the quadratic increase by coefficients which give a linear transformation. Thus, when the time points of days 11.5 through 14.5 are weighted by the following coefficients: \(-2, -1, 0, 1, 4, 9, 16\), the resulting linear regression line produces a correlation coefficient, \(r^2 = 0.99\) for +/+ points, \(n = 8\).
Fig. 2. Incorporation of $[^3]$H$]glucosamine and uronic acid concentration of macromolecules from different-aged mouse limb buds. (A, D) Chondroitin sulfate; (B, E) hyaluronic acid; (C) glycoproteins; (F) units of hyaluronidase activity from different-aged mouse limb buds. Vertical bars in (E) and (F) represent 95% confidence limit of the mean ($\bar{x} \pm t_{0.975} \times$ S.E.). In (E) 4-8 determinations were carried out for each point by the uronic acid assay and 25 determinations were made with the Reissig test after Streptomyces HAdase digestion. In (F) three determinations were made for each point. Assay variances for the incorporation of $[^3]$H$]glucosamine, uronic acid assay, and the Reissig test were all within 10% of the mean. Recovery of known quantities of HA and CS by the methods described was 90% with a variance of 3% within the mean. $\times - \times , +/ ; O - O , bp^b/bp^m$.

Although the synthetic rates in the organ culture system coincide closely (Fig. 2A), differences were observed in the incorporation of $[^3]$H$]glucosamine by 12.5-day limb mesenchyme grown in cell cultures (Table 3). Under these culture conditions the normal cells had a significantly higher incorporation rate than the mutant cells (alpha less than 0.05, two-sided $t$ test, $n = 4$). The clearer
Table 3. Incorporation of [3H]glucosamine by 12-5-day normal and brachypod limb mesenchyme in culture (sp. act. cpm/µg DNA)*

<table>
<thead>
<tr>
<th>Product</th>
<th>Genotype</th>
<th>+/+</th>
<th>bpH/bpH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycoproteins</td>
<td></td>
<td>14.01 ± 2.16†</td>
<td>17.46 ± 6.69</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td></td>
<td>42.16 ± 11.38‡</td>
<td>29.48 ± 7.39</td>
</tr>
<tr>
<td>Chondroitin sulfate</td>
<td></td>
<td>28.96 ± 4.39§</td>
<td>20.85 ± 2.98</td>
</tr>
</tbody>
</table>

* µg DNA: +/+ , 20.23 ± 3.78; bpH/bpH, 23.08 ± 6.00.
† Mean ± s.d.
‡ Statistically significant at α < 0.10.
§ Statistically significant at α < 0.05.

Table 4. Incorporation of [35S]sulfate by normal and brachypod limb buds in organ culture

<table>
<thead>
<tr>
<th>Gestational age (days)</th>
<th>Genotype</th>
<th>+/+</th>
<th>bpH/bpH</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.5–12.0</td>
<td></td>
<td>31212 ± 7204*</td>
<td>27094 ± 1485</td>
</tr>
<tr>
<td>12.25–12.75</td>
<td></td>
<td>23868 ± 6472</td>
<td>24145 ± 3012</td>
</tr>
<tr>
<td>13.0–14.0</td>
<td></td>
<td>80383 ± 15379</td>
<td>70330 ± 29363</td>
</tr>
</tbody>
</table>

* Mean ± s.d., n = 3. Data are expressed as cpm/30 limb buds.

Table 5. Incorporation of [35S]sulfate by 12-5-day normal and brachypod limb mesenchyme in culture

<table>
<thead>
<tr>
<th>Genotype</th>
<th>cpm/mg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>4990 ± 830*</td>
</tr>
<tr>
<td>bpH/bpH</td>
<td>3380 ± 370</td>
</tr>
</tbody>
</table>

* Mean ± s.d., n = 4. Difference in means is significant at P < 0.05, 2-sided t test.

expression of a difference at 12-5 days could be related to the greater availability of the radioactive precursor to cells in a monolayer as opposed to an organ culture.

The radioactive precursor, [35S]sulfate, was also used to determine sulfate incorporation into CS for normal and brachypod limb buds. It is apparent from the data in Table 4 that the variance in the incorporation of [35S]sulfate in organ culture is too large to determine if any significant difference exists. However, as observed with the incorporation of [3H]glucosamine (Table 3), the incorporation of [35S]sulfate by 12-5-day limb mesenchyme grown in cell culture was significantly lower in the mutant as compared to controls (Table 5). The lower
incorporation of both \([^{3}H]\)glucosamine and \([^{35}S]\)sulfate into CS by brachypod cell cultures suggests that a decrease in synthesis is occurring rather than a decrease in sulfation of individual CS molecules.

**Hyaluronic acid measurements**

The incorporation for HA increases until day 12.5 of gestation and then declines (Fig. 2B). After day 12.5 the radioactivity is significantly higher in the +/+ limb bud as compared to \(bp^{H}/bp^{H}\) (alpha less than 0.01, two-sided paired \(t\) test for last four time points; mean ± s.d. of +/+ minus \(bp^{H}/bp^{H}\) = 5592 ± 114 cpm, \(n = 6\)). On the other hand, the micrograms of HA at and before 12.5 days is greater in the \(bp^{H}\) but returns to normal levels after day 12.5 (Fig. 2E; alpha less than 0.05, two-sided paired \(t\) test for time points 12 and 12-5; mean ± s.d. of +/+ minus \(bp^{H}/bp^{H}\) = −9.75 ± 6.07, \(n = 4\)). Thus the levels of HA in the mutant limb bud are greater than expected from the incorporation rate over the entire time period tested and in fact are actually in excess before day 12-5. In addition, the levels of HA in both genotypes do not reflect the expected integral of the rate function as was true for CS. Instead, a pronounced dampening of HA levels occurs during days 12–13 of gestation (Fig. 2E).

It has been reported that prior to the onset of overt chondrogenesis in the chick limb there is a decreased incorporation of labeled precursor into HA concomitant with an increase in hyaluronidase (HAdase) activity (Toole, 1972). In view of these observations, experiments were carried out to determine whether similar changes in enzyme activity were also occurring in the mouse limb system. As seen in Fig. 2F there is a sharp increase in HAdase activity between days 12 and 13. The magnitude of the increase is the same for both genotypes, however, the two curves are not exactly coincident. The delay in the onset of HAdase activity in the \(bp^{H}/bp^{H}\) limb buds could contribute to the higher amounts of HA observed before 12-5 days of gestation, even though no statistical difference was demonstrated.

A series of experiments was carried out, therefore, to determine the turnover time of HA in whole limb buds which were first cultured in the presence of \([^{3}H]\)glucosamine for 6 h, then washed and further cultured in non-radioactive medium containing an excess of unlabeled glucosamine (Fig. 3). The rate of degradation of HA in the normal limb bud shows only a slight change up to 2 days of incubation, but increases sharply after the second day. When the slopes for the entire lines of both normal and brachypod are calculated, the slope for \(bp^{H}/bp^{H}\) is less negative (alpha less than 0.001) than for +/+ , indicating a slower loss of radioactive GAGs. The total turnover time was determined to be 12 days for +/+ and 13 days for \(bp^{H}/bp^{H}\).

**Equivalency of glycoprotein measurements**

An explanation other than turnover for the differences seen between genotypes in HA levels and incorporation rates is that the precursor pool of UDP-\(N-\)
Fig. 3. Turnover of glycosaminoglycans in organ culture. Twelve-day limb buds were labeled at $t_0$ with 5 $\mu$Ci/ml $[^3]$H-glucosamine for 6 h, followed by 4 days of incubation in the absence of tritiated precursor with a 50-fold excess of glucosamine. Vertical bars represent 95% confidence limit of the mean ($\bar{x} \pm t_{0.025}$ times S.E.), $n = $ four determinations for each point from three separate experiments. Slopes are significantly different $P < 0.001$, 2-sided $t$ test for regression in two populations. $\times-\times$, +/+; $O-O$, $bp^v/bp^v$.

Acetylglucosamine is larger in the mutant and thus contains relatively less radioactive glucosamine than the control. Thus, the incorporation rates when compared to +/+ would appear less than expected based on the actual levels of HA at all ages tested. An excessively large precursor pool might also be related to the occurrence of excess HA in the mutant. However, arguing against this possibility is the incorporation of $[^3]$H-glucosamine into GPs (Fig. 2C). Although GPs utilize the same precursor as HA, the radioactivity from mutant and normal are not different from day 12.5 on (mean ± s.d. of +/+ minus $bp^v/bp^v = 62$ cpm, paired t test, $n = 9$; $\alpha$ at 0.05). In addition, there is no correlation over developmental time between either $[^3]$H-HA and $[^3]$H-GP ($r^2 = 0.30$) or $[^3]$H-CS and $[^3]$H-GP ($r^2 = 0.02$, $n = 9$).

**Discussion**

The low level of CS synthesis at day 11 of gestation corresponds to the time during which the non-specific CS proteoglycan is found in the mouse limb, while the sharp increase in synthetic rate at day 12.5 corresponds to the first appear-
Glycosaminoglycan levels in normal and brachypod limbs

235

ance of the cartilage-specific molecule (Royal & Goetinck, 1977). The delay in accumulation of CS in the mutant supports previous observations made at the ultrastructural level (Duke & Elmer, 1979). The decreased CS might be related to the excess HA found before day 12-5 of gestation. It has been shown that HA is inhibitory to CS synthesis (Wiebkin & Muir, 1975, 1977; Handley & Lowther, 1976). Thus, the greater level of HA found in the mutant should act to suppress chondrogenesis. Alternatively, excess HA may block cell surface interactions thought to be important to the initiation of chondrogenesis (Toole, 1972; Levitt & Dorfman, 1974). Such a physical barrier between mutant cells may account for the observed delay in rearrangement of surface molecules that correlates with differentiation (Hewitt & Elmer, 1978). A third possible action of excess HA in the mutant involves the observation that cell adhesion is greater among the mutant cells (Duke & Elmer, 1977). This aberrant adhesion may account for the abnormal mesenchymal cell condensations observed both in vivo (Grüneberg & Lee, 1973) and in vitro (Elmer & Selleck, 1975) that occur just prior to chondrification. Since it has recently been shown that HA is an important factor in SV 3T3 cell adhesion (Underhill & Dorfman, 1978), excess HA in the mutant might be responsible for the increased adhesion of bp^11 cells. Thus, the exact manner in which HA is affecting chondrogenesis is unclear at present and could be multi-fold.

The relationship of HA and HAdase activity to cytodifferentiation is still uncertain. Some reports suggest that HA is inhibitory to chondrogenesis (Toole, 1973b; Solursh, Vaerewyck & Reiter, 1974), whereas, others indicate that it has no effect (Finch, Parker & Walton, 1978). In the present study no large differences were found between genotypes in the magnitude of HAdase activity. There was, however, a delay in the appearance of activity, and a slower turnover of labeled HA was observed for bp^11/bp^1. Excess GAG and decreased turnover was described for this mutant in the neonate also (Rhodes & Elmer, 1975). The loss of radioactivity as measured under the described conditions is not great enough to account for either the differences in the incorporation rates between the two genotypes at 12-5 days of age or the dampening of HA levels observed between days 12-13. One explanation for this is that degradation does not occur in culture at the same rate as it does in vivo. It should also be noted that the presence of at least two metabolic pools for GAGs that turn over at different rates has been previously described (Gross, Matthews & Dorfman, 1960; Lohmander, 1977). Differential labeling of such pools may give an erroneous impression of the magnitude of the turnover rate. However, based on the data presented decreased turnover is still the most probable explanation for excess HA accumulation in the mutant.

The onset of decreasing HA synthesis has the same inflection point as does increasing CS synthesis, again indicating the opposing roles of these two molecules. The pronounced dip in the HA synthetic rate at day 13 corresponds to the peak of HAdase activity. It is noteworthy that at the same time, CS appears
totally unaffected by the increase in HAdase, even though other known mamma-
lian HAdases attack both CS and HA (Saito, Yamagata & Suzuki, 1968;
Hayashi, 1978). A similar specificity was reported in regenerating newt limb
(Toole & Gross, 1971).

The regulatory mechanisms of HA levels that may be defective in brachypod
are unknown at present. The excess HA in the mutant is not due to undersul-
fated or unsulfated CS since (1) measurements of HA levels made with Strept-
tomyces HAdase, which is specific for HA, also demonstrated excess HA in
the mutant, (2) the HA peak from DEAE chromatography was equally suscep-
tible to leech-head HAdase for each genotype at the stage of excess HA in the
mutant, and (3) the $^{35}$S/$^{3}$H ratio was similar for both genotypes. Thus, this
mutation is apparently different from brachymorph, which displays a similar
limb phenotype but contains undersulfated CS (Orkin, Pratt & Martin, 1976).
In addition, the observations that CS metabolism is nearly normal except for a
delay in the onset of accumulation while HA levels are high indicate that $hp^{II}$
is unlike other cartilage mutants that display reduced skeletal elements. For
example, nanomelic chicks synthesize no cartilage-specific CS proteoglycan
(Mathews, 1967; Pennypacker & Goetinck, 1976), while the hydrocephalic
($ch^{+}$/$ch^+$) mouse shows reduced synthesis of all GAGs (Breen, Richardson,
Bondareff & Weinstein, 1973). In the achondroplastic ($cn/cn$) mouse, matrix
materials are normal (Kleinman, Pennypacker & Brown, 1977), and thus the
anomalous cell arrangements may account for the defective cartilage. In the
chondrodysplasia mutation ($cho/cho$) of the mouse, abnormal arrangement of
cells is thought to be due to abnormal matrix (Seegmiller, Fraser & Sheldon
1971). Brachypod may be related to the mouse mutation, amputated (Flint,
1977) in which the entire skeleton is affected. The abnormal cartilage is thought
to arise from increased cell adhesions that lead to defective migration of cells
into pre-cartilage condensations. A similar conclusion could be drawn for
brachypod. The abnormal HA levels might increase cell adhesion and interfere
with cell interactions of migration, and thereby lead to the incorrect or deficient
partitioning of cells into the mesenchymal condensations. In addition, the resi-
dual HA may also suppress the chondrogenic program, which consequently
results in the formation of an abnormal limb.

REFERENCES

chem. 4, 330–334.


CORSIN, J. (1975). Influence du hyaluronate et de la hyaluronidase sur la chondrogenèse

cal and physical changes in proteoglycans during development of chick limb bud chondro-
Glycosaminoglycan levels in normal and brachypod limbs 237


(Received 18 July 1979, revised 2 October 1979)