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A gradation of hyaluronate accumulation along the proximodistal axis of the embryonic chick limb bud

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SUMMARY

We are currently investigating the mechanism by which the apical ectodermal ridge (AER) of the embryonic chick limb bud exerts its negative effect on the cytodifferentiation of limb mesenchymal cells directly subjacent to it, and the mechanism by which cytodifferentiation is triggered when the cells leave the influence of the AER. Since there is a gradation of differentiation along the proximodistal axis of the limb bud, we have dissected limb buds into discrete segments along the proximodistal axis, and biochemically examined the accumulation of hyaluronate (HA) and other glycosaminoglycans (GAG) in each segment. The unspecialized subridge region of stage-25 limb buds was separated into distal (segment 1) and proximal (segment 2) regions, and the remaining proximal portion of the limb was separated into four segments (segments 3, 4, 5 and 6) along the proximodistal axis. Stage-24 limb buds were separated into corresponding regions. Since in the proximal regions of the limb (segment 3 through 6), only the cells comprising the central cores of the limb are involved in chondrogenic differentiation, the central core tissue was surgically separated from the peripheral tissue. We have found that HA is by far the predominant GAG accumulated by cells comprising the distal subridge region (representing greater than 50% of the total GAG accumulated during a 90-min labelling period with [^{3}H]glucosamine), and that there is a progressive decline in HA accumulation along the proximodistal axis. The relative and total amount of HA accumulated is highest in the distal subridge region (segment 1), intermediate in the proximal subridge region (segment 2) and lowest in the proximal central core regions of the limb (segments 3 through 6). The striking decrease in HA accumulation in the central core of segment 3 is accompanied by a striking increase in the accumulation of chondroitin sulphate, one of the major constituents of cartilage matrix. In contrast to the central core regions of segments 3 through 6, the relative and total amount of HA accumulated by the peripheral non-chondrogenic regions of these segments remains relatively high, being similar to the accumulation observed in the proximal subridge region. These results indicate that there is a gradation of HA accumulation along the proximodistal axis of both stages-24 and -25 limb buds which correlates with distance of cells from the AER and the state of differentiation of the cells.

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INTRODUCTION

The apical ectodermal ridge (AER) which extends along the distal periphery of the embryonic chick limb bud is required for the outgrowth and formation of distal limb structures by the mesodermal cells that comprise the bulk of the limb bud (see Saunders, 1977 for review). Several recent studies both *in vivo* (Stark & Searls, 1973; Summerbell, Lewis & Wolpert, 1973) and *in vitro* (Kosher, Savage & Chan, 1979*a*; Globus & Vethamany-Globus, 1976) have shown that one of the major functions of the AER is to maintain limb mesenchymal cells directly subjacent to it in an actively outgrowing, labile, undifferentiated condition, and that when the cells are freed from the AER's influence either artificially or as a result of polarized proximal to distal limb outgrowth, they are freed to commence cytodifferentiation.

We have recently demonstrated that agents that elevate cyclic AMP levels enable subridge mesenchymal cells to overcome the negative influences on cartilage differentiation and the positive influences on morphogenesis being imposed upon them by the AER (Kosher & Savage, 1980). In the presence of cyclic AMP derivatives subridge mesoderm explants cultured in the presence of the AER fail to undergo the striking proximal to distal outgrowth and contour changes characteristic of control explants, and the cessation of AER-directed morphogenesis in the presence of these agents is accompanied by the precocious chondrogenic differentiation of the mesenchymal cells (Kosher & Savage, 1980). Thus it has been suggested that when limb mesenchymal cells leave the AER's influence, their cyclic AMP content increases thus triggering chondrogenic differentiation (Kosher & Savage, 1980; see also, Kosher, Savage & Chan, 1979*b*; Ahrens, Solursh & Reiter, 1977). The increase in cyclic AMP content is thought to result from the cellular condensation and resultant intimate cell–cell interaction that occurs when subridge mesenchymal cells leave the AER's influence (Kosher & Savage, 1980).

Several other observations indicate that the cellular condensation process, which is characterized by large areas of close surface contact between adjacent cells, is a necessary prerequisite for chondrogenic differentiation. Condensation is the first morphological change that characterizes mesenchymal cells undergoing chondrogenesis *in vivo* (Thorogood & Hinchliffe, 1975) and *in vitro* (Kosher *et al.* 1979*a*); certain skeletal malformations are attributable to an impairment in the condensation process (Gruneberg & Lee, 1973); and, the chondrogenesis of dissociated limb mesenchymal cells in culture appears to be dependent upon the formation of cellular aggregates or condensations during the initial period of culture (see, for example, Umansky, 1968; Caplan, 1970). Since prior to condensation *in vivo*, subridge mesenchymal cells are separated from one another by an extensive intercellular matrix and during condensation relatively little intercellular matrix is detectable, it is possible that the intercellular matrix between subridge mesenchymal cells may be involved in main-

taining the cells undifferentiated, and that the disappearance of this matrix may be necessary to initiate condensation, and thus trigger chondrogenic differentiation. Particularly relevant to these considerations are studies by Toole (1972) on the synthesis and turnover of the glycosaminoglycan, hyaluronate, during limb development.

Toole (1972) has observed that the relative amount of hyaluronate (HA) synthesized is higher in whole chick limb buds which have not yet initiated overt cartilage formation than in older limb buds in which overt cartilage formation has begun. Furthermore, hyaluronidase activity becomes detectable at about the time a metachromatic cartilage matrix is detectable in the proximal regions of the limb bud (Toole, 1972). On the basis of these observations Toole (1972) has suggested that the synthesis of HA by limb mesenchymal cells is associated with inhibition of their differentiation, and that removal of HA may be necessary for organized chondrogenic differentiation.

This suggestion can be readily integrated with the studies indicating the AER exerts a negative effect on the differentiation of mesenchymal cells directly subjacent to it. It is conceivable, for example, that the function of the AER may be to cause the cells it is acting upon to synthesize and secrete a considerable quantity of HA, which accumulates extracellularly and maintains the cells undifferentiated. When the cells leave the AER's influence, their accumulation of extracellular HA may progressively decline (as a result of decreased synthesis, increased degradation, or both) thus initiating the condensation process that is apparently necessary to trigger chondrogenic differentiation. Since there is a gradation of differentiation along the proximodistal axis of the limb, if this hypothesis is correct, one would expect that HA would be a major component of the extracellular matrix surrounding subridge mesenchymal cells, and that there should be a gradient of HA along the proximodistal axis of the limb. To investigate this possibility, we have separated stage-24 and -25 limb buds into discrete segments along the proximodistal axis, and examined the accumulation of HA and other glycosaminoglycans in each segment. Our results indicate that there is indeed a gradation of HA accumulation along the proximodistal axis which correlates with the distance of cells from the AER and the state of differentiation of the cells.

MATERIALS AND METHODS

Preparation of tissue. Forelimb buds were removed from stage-24 and -25 (Hamburger & Hamilton, 1951) embryos of White Leghorn chicks and placed into Simms' balanced salt solution (SBSS). Stage-25 limb buds were then separated by dissection with fine knives into the various regions illustrated in Fig. 1. Photographs of the various regions into which the limb bud has been dissected are shown in Fig. 2. To begin with, the subridge region of the limb

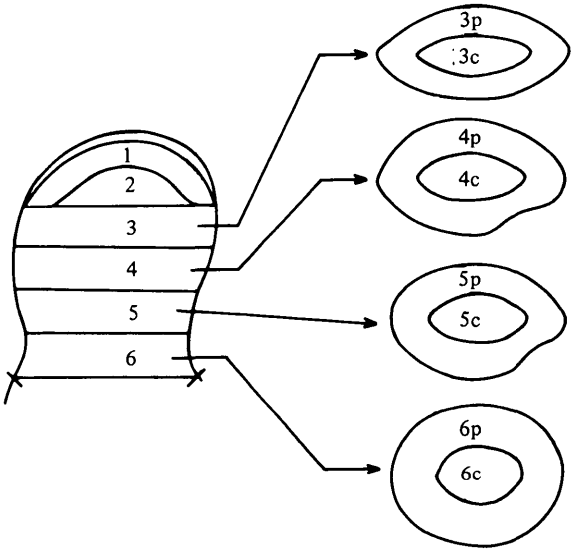

Fig. 1. The six distal-proximal segments into which stage-25 limb buds were dissected. Note that the proximal segments (3, 4, 5, and 6) in several experiments were surgically separated into central core (3c, 4c, 5c, and 6c) and peripheral (3p, 4p, 5p, and 6p) regions. Photographs of the various regions into which the limb bud has been dissected are shown in Fig. 2.

(segments 1 and 2 in Fig. 1; see also Kosher *et al.* 1979*a*) was cut away from the limb bud, and separated into distal (segment 1) and proximal (segment 2) portions. The distal subridge region (segment 1) consists of cells within approximately 0·2 mm of the AER (Figs. 1, 2*a*), and the size of the proximal subridge region (segment 2) was about 0·25 mm from the distal apex of the segment to its proximal cut edge (Figs. 1, 2*b*). Following removal of the subridge region and its dissection into segments 1 and 2, the remaining proximal portion of the limb was separated into four segments (segments 3–6) along the proximodistal axis, the proximodistal length of each of these segments being approximately 0·3 mm (Fig. 1). Stage-24 limb buds were separated into corresponding regions, but because of the shorter proximodistal length of stage-24 limb buds five, rather than six, segments were obtained. In the proximal regions of the limb (segments 3 through 6) only the cells comprising the central cores of the limb are involved in chondrogenic differentiation, whereas the peripheral tissue in these segments will differentiate into the non-chondrogenic tissues of the limb. Therefore, the central cores of segments 3–6 (3c, 4c, 5c, and 6c in Fig. 1) were surgically separated from the peripheral tissue of the segments (3P, 4P, 5P, and 6P in Fig. 1).

Photographs showing the dissection of the proximal regions (segments 3–6; Fig. 1) into central core and peripheral tissue are presented in Fig. 2*c–f*. Although the progressive chondrogenic differentiation that is occurring in the

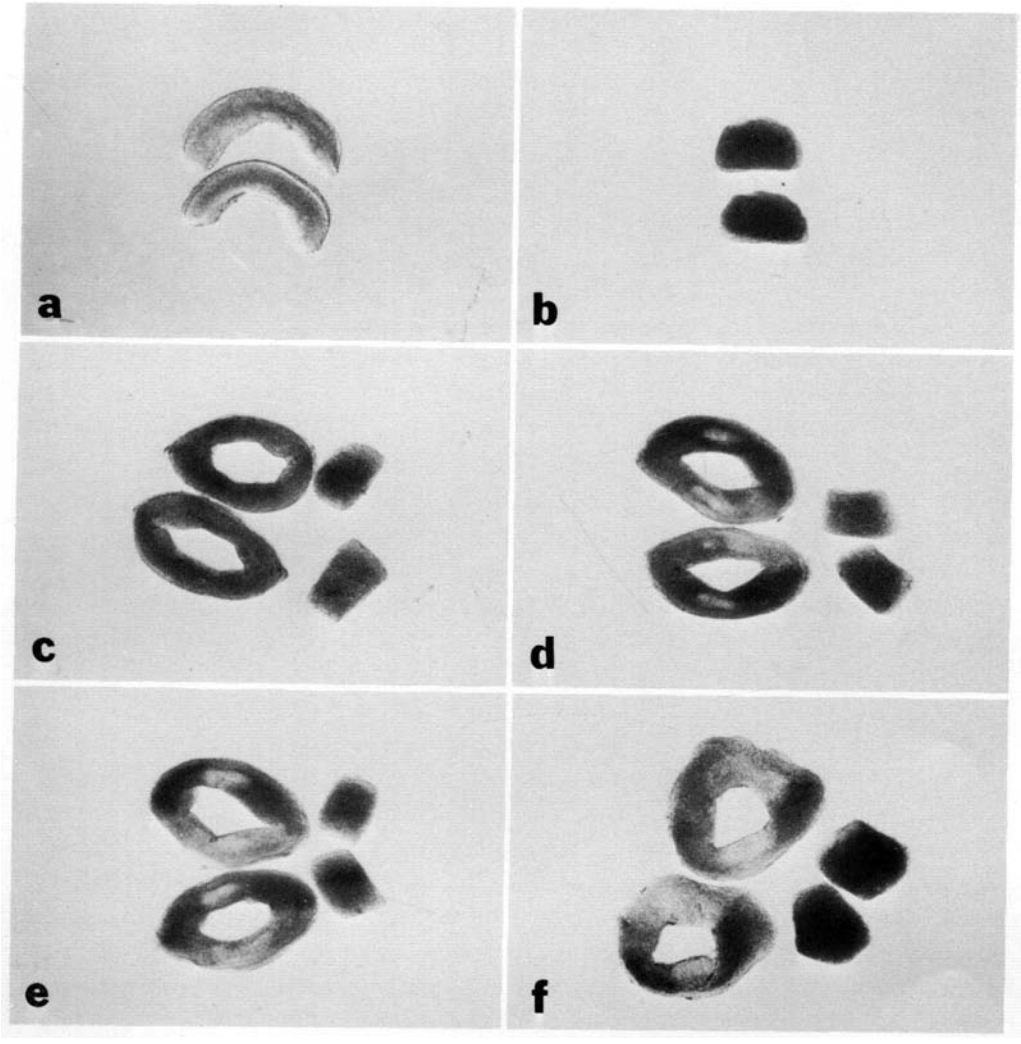

Fig. 2. Photographs of the various regions into which stage-25 limb buds have been dissected. (*a*) The distal subridge region, segment 1 (see Fig. 1). (*b*) The proximal subridge region, segment 2 (see Fig. 1). (*c*) The central core and peripheral tissue of segment 3 (see Fig. 1). (*d*) The central core and peripheral tissue of segment 4 (see Fig. 1). (*e*) The central core and peripheral tissue of segment 5 (see Fig. 1). (*f*) The central core and peripheral tissue of segment 6 (see Fig. 1).

central cores is not evident by gross examination of the living tissue, the proximo-distal gradation of differentiation is clearly evident even in the living tissue in the dorsal and ventral myogenic regions of the limb. For example, no grossly detectable differentiation is evident in the peripheral tissue of segment 3 (Fig. 2*c*), while the translucent dorsal and ventral myogenic regions of the limb become progressively more evident in segments 4–6 (Fig. 2*d–f*).

Biochemical analyses. The various regions of the limb described above were

incubated at 37 °C for 90 min in F12X medium containing 10 % foetal calf serum and 50 μCi/ml of D-[6-^{3}H](N)glucosamine (specific activity, 19 Ci/mmol; New England Nuclear), and glycosaminoglycans (GAG) were extracted from the tissues and medium as previously described (Kosher & Lash, 1975; Kosher, 1976). Briefly, the tissues and medium were sonicated and an aliquot of the sonicate removed for DNA determination (see below). The sonicates were boiled, digested with Pronase (Calbiochem), treated with ice-cold TCA, and the TCA-soluble material was extensively dialysed. The dialysates were lyophilized, and dissolved in distilled water.

The amount of [^{3}H]glucosamine-labelled hyaluronate was determined as described by Daniel, Kosher, Hamos & Lash (1974) utilizing leech hyaluronidase (hyaluronic acid B1, 3 hydrolase; Biomatrics, Inc.), an enzyme which specifically degrades hyaluronate (Meyer, Hoffman & Linker, 1960; Toole, 1976). Briefly, after treatment of an aliquot of the [^{3}H]glucosamine-labelled GAG samples with leech hyaluronidase, the amount of radioactivity associated with leech hyaluronidase-sensitive and -resistant material was determined after precipitation of undegraded GAG with cetylpyridinium chloride (CPC) (see Daniel *et al.* 1974 for details). Data were corrected for no enzyme control values, i.e. the amount of untreated material soluble in CPC. The amount of [^{3}H]glucosamine-labelled chondroitin sulphate was determined as described by Daniel *et al.* (1973) utilizing chondroitinase ABC, an enzyme which degrades the isomeric chondroitin sulphates (chondroitin 4- and 6-sulphate, and dermatan sulphate) as well as hyaluronate (Saito, Yamagata & Suzuki, 1968; Toole, 1976). Aliquots of the GAG samples were treated with chondroitinase ABC, and the amount of radio activity associated with chondroitinase-sensitive and -resistant material was determined after precipitation with CPC (Daniel *et al.* 1974). Since chondroitinase ABC degrades hyaluronate as well as the isomeric chondroitin sulphates, the amount of hyaluronate (i.e. leech hyaluronidase-sensitive GAG) was subtracted from the amount of chondroitinase-sensitive GAG to determine the amount of radioactivity associated with chondroitin sulphate.

DNA was determined by the fluorometric procedure of Brunk, Jones & James (1976) utilizing chicken blood DNA (Calbiochem) as standard.

Precursor uptake studies. Following incubation of the various limb regions in medium containing 3·0 μCi/ml of [^{3}H]glucosamine and 1·2 μCi/ml of the extracellular marker, [^{14}C]mannitol (specific activity, 51·4 mCi/mmol), the medium was removed and the amount of ^{3}H and ^{14}C radioactivity in an aliquot of it was determined. The tissues were washed once with excess SBSS, and sonicated in distilled water. One aliquot of the sonicates was removed for DNA determination, and the amount of ^{3}H and ^{14}C radioactivity in a second aliquot was determined. Since [^{14}C]mannitol does not enter cells, the amount of ^{14}C radioactivity in the sonicate was multiplied by the ratio of ^{3}H/^{14}C in the incubation medium to determine the amount of extracellular ^{3}H not removed during the SBSS wash (see Elkinton, 1947; Watkins, Cooperstein & Lazarow, 1964;

Hellman, Sehlin & Taljedal, 1971). This value was subtracted from the total amount of ^3H in the sonicate to determine the amount of [^3H]glucosamine taken up by the cells (Watkins *et al.* 1964; Hellman *et al.* 1971).

RESULTS

Table 1 shows the relative and total amounts (cpm/μg DNA) of the various [^3H]glucosamine-labelled GAG accumulated during a 90-min labelling period by the various regions of stage-25 and -24 limb buds shown in Figs. 1 and 2. The ratio of incorporation of [^3H]glucosamine into hyaluronate (HA) and chondroitin sulphate (CS) in each region is also presented in Table 1. In both stage-25 and -24 limb buds, HA is by far the predominant GAG accumulated by the distal subridge region (segment 1), and there is a progressive decline in the total and relative amount of HA between the distal subridge region and the central core of segment 3. HA accumulation (cpm/μg DNA) in the distal subridge region is about two-fold greater than in the proximal subridge region (segment 2) and about five- to six-fold greater than in the central core of segment 3 (3c). Following the decline in segment 3c, total and relative HA accumulation remains low and relatively constant in the central cores of the more proximal regions of the limb (segments 4c, 5c, and 6c at stage 25 and 4c and 5c at stage 24).

The decrease in HA accumulation between the proximal subridge region and the central core of segment 3 is accompanied by a striking (about three-fold) increase in CS accumulation (Table 1). However, only a relatively small increase (about 10–15 %) in CS accumulation (cpm/ug DNA) occurs between the distal and proximal subridge regions. Following the striking increase in segment 3c, CS accumulation remains high in the central cores of more proximal limb regions, although there is a gradual decline in the total amount of CS accumulated in the extreme proximal central cores. The gradual decline in the amount of CS accumulated during a 90-min labelling period between segment 3c and more proximal central core regions in which chondrogenic differentiation is more advanced is not particularly surprising. One might expect the rate of synthesis of cartilage matrix components to be more rapid during the early stages of differentiation when cartilage matrix is just beginning to be accumulated than later in the process when a considerable quantity of matrix has already been accumulated. In fact, Lavietes (1971) has observed that the rate of CS synthesis by dissociated chondrocytes in monolayer culture is highest during the initial period of culture when the cells are just beginning to accumulate a cartilage matrix, and that the rate of CS synthesis gradually decreases during the later periods of culture as the cells progressively accumulate more cartilage matrix.

In contrast to the striking changes in total and relative HA and CS accumulation that occur between the proximal subridge region and the central core areas of more proximal limb regions, the pattern of GAG accumulation in the

Table 1. *Relative and total (cpm/μg DNA) amounts of the various [^{3}H]glucosamine-labelled GAG accumulated by various regions of stage-25 and -24 limb buds**

Developmental stage	Limb region (see Figs. 1 and 2)	HA†		CS‡		Other GAG§		HA/CS ratio
		Cpm/μg DNA	%	Cpm/μg DNA	%	Cpm/μg DNA	%	
25	1	1469	52·8	466	16·8	845	30·4	3·14
	2	752	39·6	542	28·5	606	31·9	1·39
	3c	239	8·5	1798	63·5	794	28·0	0·13
	3p	536	33·6	508	31·8	552	34·6	1·06
	4c	261	10·7	1530	62·8	647	26·5	0·17
	4p	464	38·0	366	30·0	390	32·0	1·27
	5c	157	9·0	1192	68·3	396	22·7	0·13
	5p	452	40·0	325	28·7	354	31·3	1·39
	6c	194	13·6	856	60·1	375	26·3	0·23
	6p	242	36·4	207	31·2	215	32·4	1·17
24	1	1834	54·1	618	18·2	940	27·7	2·97
	2	1092	44·1	655	26·5	729	29·4	1·66
	3c	403	14·1	1653	57·8	804	28·1	0·24
	3p	478	38·0	345	27·4	436	34·6	1·39
	4c	268	12·2	1351	61·6	576	26·2	0·20
	4p	488	41·3	336	28·4	359	30·3	1·45
	5c	197	17·9	648	58·9	256	23·2	0·30
	5p	306	41·3	209	28·2	226	30·5	1·46

* Each value represents the average of two separate determinations.
† Leech hyaluronidase-sensitive GAG.
‡ Amount of chondroitinase ABC-sensitive GAG minus the amount of leech hyaluronidase-sensitive GAG.
§ Chondroitinase ABC-resistant GAG.

Table 2. *Accumulation of the various GAG relative to the amount of precursor uptake in the various regions of stage-25 limb buds*

Limb region (see Figs. 1 and 2)	Normalized precursor uptake*	accumulation (cpm/ug DNA)/normalized precursor uptake†		
		HA	CS	Other GAG
1	1·00	1469	466	845
2	0·83	906	653	730
3c	0·59	405	3047	1346
3p	0·67	800	758	824
4c	0·46	567	3326	1407
4p	0·56	829	654	696
5c	0·48	327	2483	825
5p	0·56	807	580	632
6c	0·50	388	1712	750
6p	0·56	432	370	384

* The amount of precursor uptake (cpm/μg DNA) in each limb region was divided by the amount of uptake in segment 1 (1690 ± 94 cpm/μg DNA, $n = 3$). Normalized rather than absolute precursor uptake values are utilized, since in the uptake experiments 3·0 μCi/ml of [^{3}H]glucosamine was used rather than the 50 μCi/ml used in the incorporation studies shown in Table 1. It should be noted, however, that in an uptake experiment in which 50 μCi/ml of [^{3}H]glucosamine was utilized, virtually the same relative differences in precursor uptake in different limb regions were observed as when 3·0 μCi/ml was utilized.

† The incorporation values in Table 1 (cpm/μg DNA) were divided by the normalized precursor uptake values shown above.

proximal, non-chondrogenic peripheral regions (3p, 4p, 5p, and 6p) is very similar to that observed in the proximal subridge region (Table 1). Thus, total HA accumulation during the 90 min labelling period is in general about twofold greater in the proximal peripheral regions than in the chondrogenic, central core regions, and CS accumulation is three- to fourfold greater in the central core tissue than in the peripheral tissue.

It should be mentioned that in one experiment the pattern of GAG accumulation by the distal subridge region was examined in the presence and absence of the small amount of ectodermal tissue that surrounds the segment, and virtually no difference in the pattern was observed, i.e. the HA/CS ratio was 2·55 in the presence of ectoderm and 2·44 in its absence. Thus, the small amount of ectodermal tissue makes little, if any detectable contribution to the pattern of GAG accumulation.

The differences in total accumulation of various GAG in different limb regions might conceivably reflect not only differences in synthetic capacity, but also perhaps differences in the uptake and availability of the radioactive precursor. To investigate the latter possibility, total [^{3}H]glucosamine uptake per μg DNA in the various regions of stage-25 limbs was examined as described in Materials and Methods. As shown in Table 2, distinct differences in total precursor uptake were observed in different limb regions. However, virtually

the same overall pattern of GAG accumulation as already described is evident when the total accumulation (cpm/μg DNA) of the various GAG in different limb regions is expressed relative to the total precursor uptake in that region (Table 2), i.e. in the distal subridge region HA accumulation relative to the amount of precursor uptake is 60–70 % greater than in the proximal subridge region and three- to fourfold greater than in the central core of segment 3 and more proximal central core regions; there is a striking increase (about fourfold) in CS accumulation relative to total precursor uptake in segment 3c and more proximal central core regions, while only a relatively small increase occurs between the distal and proximal subridge regions; and, CS accumulation relative to precursor uptake is about fourfold greater in the proximal central core regions than in the proximal peripheral regions, and HA accumulation relative to precursor uptake is about twofold greater in the proximal peripheral regions than in the proximal, central core regions. Thus the differences in HA and CS accumulation in different limb regions appear to reflect differences in synthetic capacity, and not simply differential precursor uptake.

DISCUSSION

The results of the present investigation indicate that there is a gradation of HA accumulation along the proximodistal axis of embryonic chick limb buds which correlates with distance of cells from the AER and the state of differentiation of the cells. The relative and total amount of HA accumulated is highest in the distal subridge region (segment 1), intermediate in the proximal subridge region (segment 2), and lowest in the proximal central core regions of the limb (segments 3 through 6). The striking decrease in HA accumulation in the central core of segment 3 is accompanied by a striking increase in the accumulation of CS, one of the major constituents of cartilage matrix. In contrast to the proximal central core regions, the relative and total amount of HA accumulated by the peripheral non-chondrogenic areas of proximal regions of the limb in general remains relatively high, being similar to the accumulation observed in the proximal subridge region. The differences in HA and CS accumulation we have observed biochemically in various regions of the limb correlate well with histochemical and autoradiographic studies. Sugrue (1979) has obtained histochemical evidence that HA is present in high amounts in the subridge region of rat limb buds, and is greatly reduced in the chondrogenic central core. Searls (1965) has observed autoradiographically that the incorporation of [^{35}S]sulphate into sulphated GAG is relatively low in the subridge region of the chick limb bud, and very high in the region of the limb corresponding to the central core of segment 3, in which we biochemically observe greatly enhanced chondroitin sulphate accumulation.

Since in the present studies a relatively short (90 min) labelling period was utilized, it is likely that the observed decrease in HA accumulation along the

proximodistal axis reflects primarily a progressive decrease in synthetic capacity. It is possible, however, that the greatly reduced accumulation of HA observed in the proximal subridge region and central core of segment 3 during the 90-min labelling period results partially from a more rapid rate of degradation of newly synthesized HA in these regions, as well as decreased synthetic capacity. In this regard, Toole (1972) has observed a correlation between a decrease in the HA/CS ratio in whole limb buds and an increase in the activity of the enzyme, hyaluronidase. Therefore, experiments are in progress to determine the rate of degradation of HA and hyaluronidase activity in the various regions of the limb described in this study. A likely possibility is that, as cells become located progressively farther from the AER, their rate of synthesis of HA may progressively decrease and concomitantly pre-existing extracellular HA may be removed as a result of an activation of or an increase in activity of hyaluronidase.

The gradation of HA accumulation along the proximodistal axis correlates not only with the distance of cells from the AER, but also with changes in the extent of cell–cell contact. The tissue comprising the distal subridge region in which HA accumulation is the greatest consists of a loosely constructed network of non-specialized mesenchymal cells separated from one another by extensive intercellular spaces with cellular contacts being primarily via filapodia (Thorogood & Hinchliffe, 1975; Kosher & Grasso, in preparation). In contrast, the central core of segment 3 in which HA accumulation is minimal consists predominantly of cells which are in the condensation phase of chondrogenesis, i.e. there is broad and extensive close surface contact between the cells and a corresponding large reduction in the amount of extracellular space (Thorogood & Hinchliffe, 1975; Kosher and Grasso, in preparation). In the central core regions of more proximal segments of the limb (segment 4c, 5c and 6c), the cells lose their close surface associations as they begin to become progressively separated from one another by cartilage matrix (Thorogood & Hinchliffe, 1975; Kosher and Grasso, in preparation).

The correlations between changes in HA accumulation, distance of cells from the AER, and changes in the extent of cell–cell contacts are of interest for a number of reasons. First of all, as previously described, one of the major functions of the AER is to maintain mesenchymal cells directly subjacent to it (cells in the distal subridge region) in a labile, undifferentiated condition. The results presented in the present study are consistent with the possibility that the AER may do so at least in part by causing the cells it is acting upon to synthesize and secrete a considerable quantity of HA. The extracellular HA which thus accumulates may be involved in maintaining the cells undifferentiated perhaps by simply maintaining the cells physically separated from one another and thus preventing the cellular condensation and intimate cell–cell interaction that appears to be necessary to trigger chondrogenic differentiation (see Introduction). As has been pointed out by Toole (1976) the physical-chemical properties of HA make it an ideal candidate for physically separating cells, and

thus preventing extensive cell–cell interactions. In fact, Toole, Jackson & Gross (1972) have demonstrated that exogenous HA inhibits the aggregation of dissociated stage-26 chick limb-bud cells in monolayer culture. Furthermore, exogenous HA exerts an inhibitory effect on proteoglycan synthesis and cartilage matrix deposition by mature chondrocytes in suspension culture (Wiebkin & Muir, 1973) and in monolayer culture (Solursh, Vaerewyck & Reiter, 1974). Thus, extracellular HA has the potential to maintain subridge mesenchymal cells undifferentiated not only by preventing cellular condensation, but also by feeding back upon the mesenchymal cells and thus inhibiting their synthesis of cartilage matrix components.

If extracellular HA is indeed involved in physically separating subridge mesenchymal cells from one another, a drastic reduction in its synthesis and concomitant removal of previously synthesized extracellular HA by hyaluronidase might be expected to result in the cellular condensation necessary to trigger chondrogenic differentiation. The correlation between the progressive decline in HA accumulation along the proximodistal axis of the limb and corresponding changes in the extent of cell–cell contact are perfectly consistent with this possibility. That the reduction in HA accumulation along the proximodistal axis may be causally related to the initiation of the cellular condensation process is supported by the recent finding of Shambaugh & Elmer (1980) that the limbs of brachypod mouse mutants, whose skeletal malformations are attributed to an impairment in the condensation process, exhibit a delay in the reduction of HA synthesis that occurs in normal limbs on day 12.5 of gestation, and this delay correlates with the reduced cellular condensations in the mutant limb.

In summary, on the basis of all of the above observations and our previous studies, we suggest the following mechanism concerning the relationship between the function of the AER, HA, cellular condensation, and cyclic AMP in limb morphogenesis and differentiation. The AER synthesizes and secretes a molecule(s) which maintains subjacent mesenchymal cells in a labile, undifferentiated condition. The function of the putative AER 'inhibitory' molecule(s) may be to cause the mesenchymal cells it is acting upon to synthesize and secrete a considerable quantity of HA. The extracellular HA which thus accumulates may maintain the subridge mesenchymal cells physically separated from one another and thus prevent the cellular condensation that is required to trigger chondrogenic differentiation. In addition, extracellular HA might conceivably feed back upon the mesenchymal cells and thus inhibit their synthesis of cartilage matrix components. As cells become located progressively further from the AER and its inhibitory influence as a result of polarized proximal to distal outgrowth, their synthesis of HA may progressively decline and concomitantly preexisting extracellular HA may be removed as a result of an activation of or an increase in activity of hyaluronidase. As a result, the mesenchymal cells undergo condensation, and the resulting interaction between molecules on adjacent cell surfaces results in an elevation of the cyclic AMP

content of the cells. As a result of the elevation in cyclic AMP content, chondrogenic differentiation is triggered.

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