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Studies on the possible role of cyclic AMP in limb morphogenesis and differentiation

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SUMMARY

Recent studies indicate that one of the major functions of the apical ectodermal ridge (AER) of the embryonic chick limb bud is to maintain mesenchymal cells directly subjacent to it (i.e. cells extending 0·4–0·5 mm from the AER), in a labile, undifferentiated condition, and that when mesenchymal cells are freed from the AER's influence either artificially or as a result of normal polarized proximal to distal limb outgrowth, they are freed to commence cyto-differentiation. In a preliminary attempt to investigate at a molecular level the mechanism by which the AER exerts its 'negative' effect on the cytodifferentiation of subjacent mesenchymal cells, we have examined the effect of a variety of agents that elevate cyclic AMP levels on the morphogenesis and differentiation of the unspecialized subridge mesoderm in an organ culture system. *In vitro* in the presence of the AER, undifferentiated subridge mesoderm explants undergo remarkably normal morphogenesis characterized primarily by progressive polarized proximal to distal outgrowth and changes in the contour of the developing explant. In the presence of cyclic AMP derivatives, explants fail to undergo the polarized outgrowth and contour changes characteristic of control explants. In fact, in the presence of dibutyryl-cyclic AMP and theophylline, AER-directed morphogenesis essentially ceases during the first day of culture. The cessation of AER-directed morphogenesis in the presence of cyclic AMP derivatives is accompanied by the histochemically and biochemically detectable precocious chondrogenic differentiation of the subridge mesenchymal cells. In control explants, cartilage differentiation only occurs in those proximal cells of the explant which gradually become located greater than 0·4–0·5 mm from the AER. In contrast, in the presence of cyclic AMP derivatives, cartilage differentiation by cells within 0·4–0·5 mm of the AER is detectable from the first day of culture, and by the third day cartilage formation has occurred throughout the entire explant. Overall, these results indicate that elevating the cyclic AMP content of the subridge mesenchymal cells enables the cells to overcome negative influences on cytodifferentiation and the positive influences on morphogenesis being imposed upon them by the AER. On the basis of this observation and previous studies, a testable model on the role of cyclic AMP in limb morphogenesis and differentiation is proposed.

INTRODUCTION

The apical ectodermal ridge (AER) which is a cap of pseudostratified columnar epithelium that extends around the distal periphery of the embryonic chick limb bud exerts a profound influence on the mesodermal cells that constitute the bulk of the limb bud. Surgical removal of the AER results in the formation

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of limbs possessing severe distal deficiencies (Saunders, 1948; Summerbell, 1974) and grafting an extra AER onto the mesodermal cells of the limb bud results in the formation of limbs possessing distal duplications (Saunders and Gasseling, 1968; Zwilling, 1956*a*, Saunders, Gasseling & Errick, 1976). Furthermore, the spontaneous degeneration of the AER in the *wingless* mutant chick embryo is accompanied by the formation of limbs possessing severe terminal deficiencies, and the spontaneous appearance of a second AER on the dorsal side of the limb bud in the mutant *eudiplopodia* is accompanied by the formation of limbs possessing supernumerary distal structures (Goetinck, 1966). The mechanism by which the AER exerts its profound effect on the outgrowth and formation of distal limb structures by the mesoderm cells has, however, remained obscure.

During the earliest stages of embryonic chick limb development (stage 16–22; Hamburger & Hamilton, 1951) the mesodermal cells of the limb bud appear to be a population of non-specialized mesenchymal cells that are virtually identical to one another ultrastructurally (Searls, Hilfer & Mirow, 1972; Thorogood & Hinchliffe, 1975) and biochemically (Searls, 1965*a*, *b*; Linsenmayer, Toole & Trelstad, 1973). During the fourth–fifth day of development (stages 23–25) the mesenchymal cells in the proximal central core of the limb bud (the so-called chondrogenic area) undergo a variety of ultrastructural and biochemical changes indicative of their differentiation into cartilage (Searls, 1965*a*, *b*; Searls *et al.* 1972; Thorogood & Hinchliffe, 1975), and mesenchymal cells in the dorsal and ventral proximal regions of the limb (the so-called myogenic areas) acquire the ultrastructural characteristics of muscle-forming and connective tissue cells (Hilfer, Searls & Fonte, 1973). However, as the mesenchymal cells in the proximal chondrogenic and myogenic areas of the limb initiate differentiation at stages 22–23, those mesenchymal cells directly subjacent to the AER, i.e. cells extending 0·4–0·5 mm from the AER, retain the characteristics of unspecialized mesenchymal cells, and exhibit no signs of differentiation (Searls, 1965*a*; Searls *et al.* 1972; Stark & Searls, 1973). During subsequent stages of development as the limb undergoes AER-directed polarized outgrowth in a proximal to distal direction, the size of the non-differentiating subridge region remains constant (Searls, 1965*a*, 1973; Stark & Searls, 1973), although the cells in the subridge region double in number every 11–13 h (Janners & Searls, 1970). Apparently, therefore, when polarized limb growth and division in the subridge region causes cells to become located greater than 0·4–0·5 mm from the AER, the cells become part of the proximal chondrogenic and myogenic areas and initiate cytodifferentiation (Searls, 1965*a*, 1973; Stark & Searls, 1973).

On the basis of the above observations, it has been suggested that one of the functions of the AER may be to maintain mesenchymal cells in the subridge region in a labile, undifferentiated condition (Stark & Searls, 1973, see also Summerbell, Lewis & Wolpert, 1973). We have recently obtained direct evidence supporting this hypothesis by studying in an organ culture system the morpho-

genesis and differentiation of the unspecialized subridge mesoderm of the embryonic chick wing bud in the presence and absence of the AER (Kosher, Savage & Chan, 1979*a*). In the presence of the AER, subridge mesoderm explants undergo remarkably normal morphogenesis characterized primarily by progressive polarized proximal to distal outgrowth, and changes in the contour of the developing explant (Kosher *et al.* 1979*a*). As cells of the explant become located greater than 0·4–0·5 mm from the AER as a result of polarized outgrowth, they concomitantly initiate chondrogenic differentiation, whereas cells remaining 0·4–0·5 mm from the AER retain the characteristics of unspecialized mesenchymal cells showing no indications of differentiation into cartilage or any other tissue type (Kosher *et al.* 1979*a*). In contrast, when the subridge mesoderm is cultured in the absence of the AER, the cells fail to undergo morphogenesis and rapidly and precociously initiate chondrogenic differentiation (Kosher *et al.* 1979*a*). These results indicate that the AER maintains limb mesenchymal cells directly subjacent to it in a labile, undifferentiated condition, and that when mesenchymal cells are freed from the AER's influence either artificially or as a result of polarized proximal to distal outgrowth, they are freed to commence cytodifferentiation (Kosher *et al.* 1979*a*).

We have recently initiated a series of experiments designed to investigate at a molecular level the mechanism by which the AER exerts its 'negative' effect on the cytodifferentiation of subjacent limb mesenchymal cells, and the mechanism by which cytodifferentiation is triggered when cells are freed from the AER's influence. In our initial investigation in this regard, we demonstrated that a variety of agents that elevate cyclic AMP levels stimulate the already precocious chondrogenic differentiation that subridge mesoderm explants undergo in organ culture in the absence of the AER (Kosher, Savage & Chan, 1979*b*). For example, the dibutyryl- and 8-hydroxy derivatives of cyclic AMP elicit a dose-dependent increase in the rate and amount of cartilage matrix formation and a corresponding two- to three-fold increase in sulfated glycosaminoglycan accumulation by subridge mesoderm explants cultured in the absence of the AER (Kosher *et al.* 1979*b*). On the basis of these observations and our previous studies, we suggested that when limb mesenchymal cells are removed from the influence of the AER, their cyclic AMP content increases thus triggering chondrogenic differentiation (Kosher *et al.* 1979*b*). The AER via molecule(s) produced by it may, therefore, exert its negative effect on the cytodifferentiation of subjacent mesenchymal cells by preventing either directly or indirectly this elevation in cyclic AMP content from occurring (Kosher *et al.* 1979*b*).

One manner in which the above hypothesis can be tested is to examine the effect of agents that elevate cyclic AMP levels on the morphogenesis and differentiation of the subridge mesoderm cultured in the presence of the AER, a condition under which the subridge mesenchymal cells would normally undergo AER-directed polarized proximal to distal outgrowth and be maintained undifferentiated (Kosher *et al.* 1979*a*). If the hypothesis is correct, one would

predict that precociously elevating the cyclic AMP content of the subridge mesenchymal cells cultured in the presence of the AER might impair or prevent their AER-directed polarized outgrowth and concomitantly elicit precocious chondrogenic differentiation. Accordingly, in the present investigation, we demonstrate that agents that elevate cyclic AMP levels do indeed inhibit the morphogenesis of subridge mesenchymal cells cultured in the presence of the AER and overcome the 'negative' effect of the AER on cytodifferentiation.

MATERIALS AND METHODS

Materials. N^6,O^2-dibutyryl adenosine 3′,5′ cyclic monophosphoric acid (Grade II, sodium salt), 8-hydroxyadenosine 3′,5′ cyclic monophosphoric acid, adenosine 5′-monophosphoric acid (Type II, sodium salt), N^2,O^2-dibutyryl guanosine 3′,5′ cyclic monophosphoric acid (sodium salt) and theophylline were purchased from Sigma Chemical Company.

Preparation of cultures. Distal wing-bud tips composed of the subridge mesoderm capped by the AER and surrounded dorsally and ventrally by ectoderm were cut away from stage-25 (Hamburger & Hamilton, 1951) embryos of White Leghorn chicks as previously described (Kosher *et al.* 1979*a*). The size of the excised distal wing-bud tips was 0·4–0·5 mm from the distal apex of the tissue to the proximal cut edge (see Kosher *et al.* (1979*a*) for a photograph). Explants were cultured on nutrient agar containing F12X medium supplemented with 10% fetal calf serum and 1% Bovine Albumin Fraction V as previously described (Kosher *et al.* 1979*a*). For the biochemical studies described below, the nutrient agar and feeding medium contained 5 μCi/ml of $H_2{}^{35}SO_4$ (carrier free; New England Nuclear), and was supplemented with cyclic nucleotides or theophylline by directly dissolving the agents in the medium at the concentrations indicated in Results, usually 1·0 mM.

Histological and histochemical procedures. At various times following the initiation of culture, explants were fixed in 4% formalin containing 0·5% cetylpyridinium chloride, dehydrated, embedded in paraffin, sectioned at 5 μm, and routinely stained with hematoxylin and eosin. In addition, in order to histochemically examine the accumulation of sulfated glycosaminoglycans by the explants, sections were stained as described by Yamada (1970) with 0·5% Alcian blue, pH 1·0.

Biochemical analysis. Glycosaminoglycans were extracted from explants continuously exposed to [^{35}S]sulfate by procedures previously described (Kosher, 1976). DNA was determined by a micromodification of the procedures of Abraham, Scaletta & Vaughn (1972) and Richards (1974).

RESULTS

Effect of cyclic AMP derivatives on AER-directed in vitro *morphogenesis of the subridge mesoderm.* The typical gross *in vitro* morphogenesis that subridge

mesoderm explants undergo during the first 3 days of culture in the presence of the AER is shown in Fig. 1(*a*, *b*, *c*) (see Kosher *et al.* 1979*a*, fig. 1, for a photograph of a freshly excised subridge mesoderm explant just prior to its being placed in organ culture). The AER-directed morphogenesis the subridge mesoderm undergoes *in vitro* is characterized primarily by progressive polarized proximal to distal outgrowth and changes in the contour of the developing explant (Fig. 1*a*, *b*, *c*). In fact, as previously described (Kosher *et al.* 1979*a*), the sequence of morphogenesis the subridge mesoderm undergoes *in vitro* is very similar to the sequence of morphogenesis distal wing-bud tips undergo *in vivo* during stages 25 through 29.

The gross morphogenesis that subridge mesoderm explants undergo in the presence of 1·0 mM dibutyryl-cyclic AMP (db-cAMP) and in the presence of 1·0 mM db-cAMP and 1·0 mM of the phosphodiesterase inhibitor theophylline is shown in Fig. 1(*d*, *e*, *f*) and Fig. 1(*g*, *h*, *i*) respectively. Although no dramatic effects on gross morphogenesis are yet detectable during the first day of culture, the inhibitory effect of cyclic AMP derivatives on AER-directed morphogenesis is strikingly evident during the second and third days of culture. In a very reproducible and consistent manner, cyclic AMP-treated explants fail to undergo the polarized outgrowth and contour changes characteristic of control explants. In 1·0 mM db-cAMP-treated explants only a small amount of tissue directly subjacent to the AER undergoes a slight amount of atypical, asymmetric outgrowth between the first and second day of culture (Fig. 1*e*), and virtually no additional outgrowth occurs between the second and third days (Fig. 1*f*). When theophylline is added along with db-cAMP, inhibition of morphogenesis is even more striking, in that between the first and second day of culture, virtually no polarized outgrowth or contour changes occur (Fig. 1*h*), i.e. AER-directed morphogenesis of db-cAMP and theophylline-treated explants essentially ceases between the first and second day of culture.

It is also noteworthy that in control explants throughout the period of culture, tissue within 0·4–0·5 mm of the AER has a homogeneous translucent appearance, whereas tissue in those proximal regions of the explant that have become located greater than 0·4–0·5 mm from the AER has a considerably denser appearance (Fig. 1*b*, *c*). In contrast, in db-cAMP and theophylline-treated explants by the third day of culture virtually the entire explant is composed of dense-appearing tissue, and none of the more translucent-appearing tissue characteristic of the subridge region of control explants is seen (Fig. 1*i*). The denser appearance of the tissue seen in the proximal regions of control explants and throughout db-cAMP and theophylline-treated explants is a manifestation of significant histological and biochemical changes that are occurring in these cells (see following section of Results).

It also is of interest that db-cAMP has no noticeable effect on the gross morphology of the AER. Note in Fig. 1 that the AER in treated explants remains thickened and similar in appearance to the AER of control explants. It does

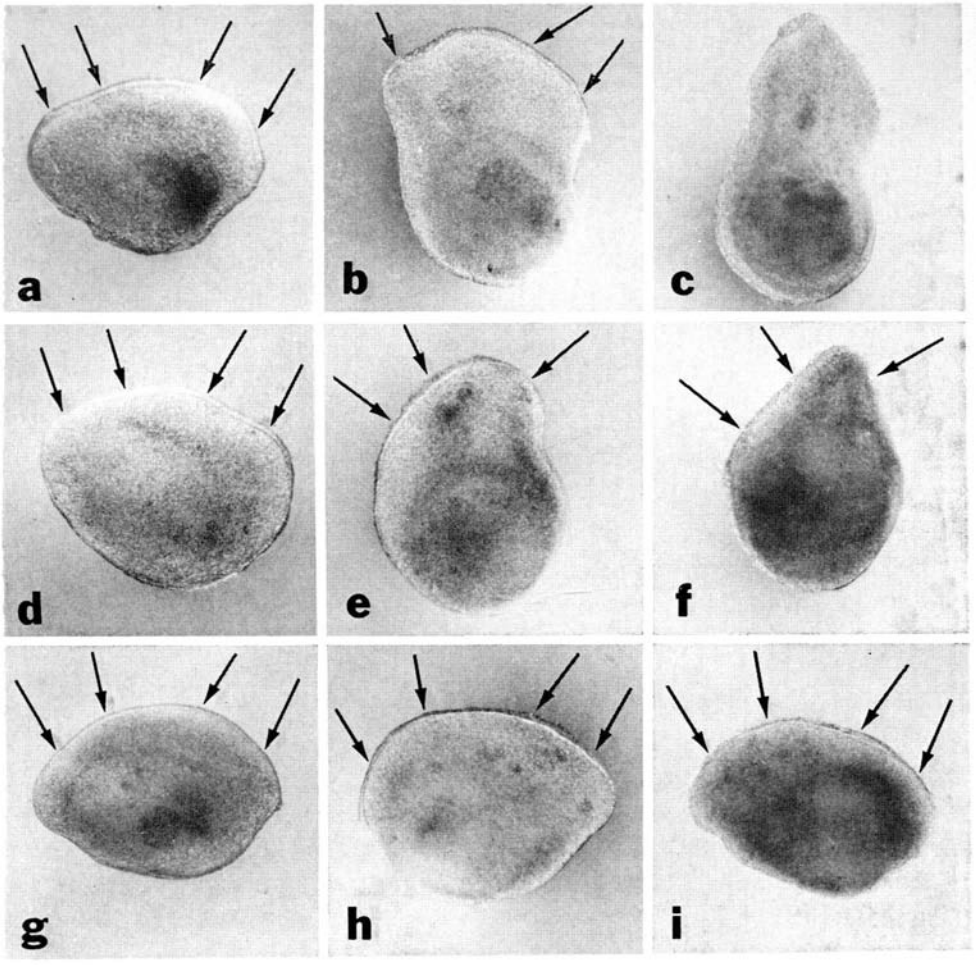

Fig. 1. The gross *in vitro* morphogenesis of control (*a*, *b*, *c*), 1·0 mm db-cAMP-treated (*d*, *e*, *f*) and 1·0 mM db-cAMP and 1·0 mM theophylline-treated (Fig. 1 *g*, *h*, *i*) subridge mesoderm explants.

(*a*)–(*c*) Living control explants during the first (*a*), second (*b*) and third (*c*) days of culture. The AER is indicated by arrows. Note the progressive polarized proximal to distal outgrowth and contour changes that occur during the period of culture. × 70.

(*d*)–(*f*) Living 1·0 mM db-cAMP-treated explants during the first (*d*), second (*b*) and third (*c*) days of culture. The AER is indicated by arrows. Note that in contrast to control explants (Fig. 1 *a*, *b*, *c*) only a small amount of atypical, asymmetric outgrowth occurs between the first (*d*) and second (*e*) days of culture in the presence of db-cAMP, and virtually no additional outgrowth occurs between the second (*e*) and third days (*f*). × 70.

(*g*)–(*i*) Living 1·0 mM db-cAMP and 1·0 mM theophylline-treated explants during the first (*g*), second (*h*) and third (*i*) days of culture. The AER is indicated by arrows. Note that virtually no outgrowth or contour changes occur between the first (*g*) and subsequent (*h*, *i*) days of culture. × 70.

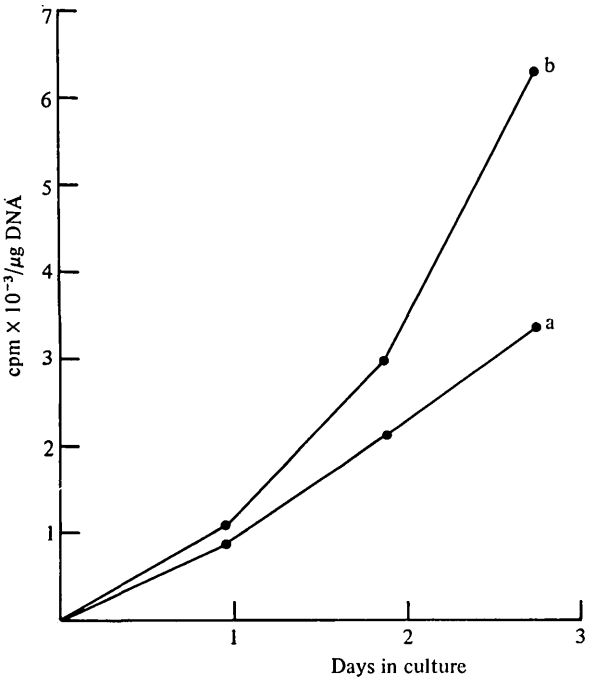

Fig. 2. Accumulation of [^{35}S]sulfate-labeled glycosaminoglycans (GAG) by control subridge mesoderm explants in the presence of the AER (*a*) and by explants cultured in medium supplemented with 1·0 mM db-cAMP (*b*).

not flatten and undergo the degenerative changes characteristic of AER's that have lost their effect on subjacent mesodermal cells (see Kosher *et al.* 1979*a*).

The inhibitory effect of db-cAMP on AER-directed morphogenesis is dose-dependent; 2·0 mM db-cAMP has a greater inhibitory effect on morphogenesis than does 1·0 mM db-cAMP (inhibiting morphogenesis to about the same extent as 1·0 mM db-cAMP and 1·0 mM theophylline), while 0·1 mM has no perceptible effect on morphogenesis. The effect is not limited to the dibutyryl derivative of cyclic AMP, since 8-hydroxy-cyclic AMP (8-OH-cAMP) also elicits a striking inhibition of morphogenesis. However, the inhibitory effect appears to be limited to cyclic AMP derivatives, since neither the non-adenosine cyclic nucleotide, db-cGMP, nor the non-cyclic adenosine nucleotide, 5′-AMP, have any perceptible effect on morphogenesis.

Effect of cyclic AMP derivatives on the differentiation of the subridge mesoderm in the presence of the AER. Results described thus far indicate that elevating the cyclic AMP content of the subridge mesenchymal cells cultured in the presence of the AER inhibits AER-directed polarized proximal to distal outgrowth. In initial experiments to determine if agents that elevate cyclic AMP levels concomitantly elicit precocious chondrogenic differentiation of the subridge

Table 1. *Effect of cyclic and noncyclic nucleotides on the accumulation of* [^{35}S] *sulfate-labeled glycosaminoglycans by subridge mesoderm explants cultured in the presence of the AER*

		1 day		2 days		3 days	
Experiment	Treatment	cpm/μg DNA	Treated /control	cpm/μg DNA	Treated /control	cpm/μg DNA	Treated /control
1	0·1 mM db-cAMP	1058	1·03	2903	1·15	4116	1·17
	None	1024	—	2531	—	3518	—
2	1·0 mM db-cAMP	1066	1·19	2961	1·41	6268	1·88
	None	896	—	2102	—	3328	—
3	2·0 mM db-cAMP	1735	1·39	4512	1·75	8450	2·30
	None	1248	—	2579	—	3676	—
4	1·0 mM db-cAMP+ 1·0 mM theophylline	1191	1·31	3124	1·88	5614	2·28
	None	908	—	1659	—	2464	—
5	1·0 mM theophylline	964	1·06	1904	1·13	2981	1·04
	None	909	—	1679	—	2863	—
6	1·0 mM 8-OH-cAMP	1601	1·21	4678	2·39	4591	1·85
	None	1321	—	1960	—	2488	—
7	1·0 mM db-cGMP	840	0·90	1815	1·00	3988	1·01
	None	932	—	1818	—	3935	—
8	1·0 mM 5′-AMP	682	0·96	1874	1·14	2942	1·19
	None	709	—	1644	—	2472	—

mesenchymal cells, we examined the accumulation of sulfated glycosaminoglycans (GAG), one of the major constituents of cartilage matrix, by control and cAMP-treated subridge mesoderm explants. Previous studies have established that there is an excellent correlation between the amount of sulfated GAG accumulated by subridge mesoderm explants and the amount of histologically and histochemically detectable hyaline cartilage matrix formed by the explants (Kosher *et al.* 1979*a*, *b*; see also Kosher & Lash, 1975; Kosher, 1976).

Figure 2 demonstrates the accumulation of [^{35}S]sulfate-labeled GAG by control subridge mesoderm explants in the presence of the AER and by explants cultured in the presence of 1·0 mM db-cAMP. From the first day of culture onward there is a progressive increase in the amount of sulfated GAG accumulated by db-cAMP-treated explants compared to controls, until by the third day of culture db-cAMP-treated explants have accumulated approximately two-fold more sulfated GAG than control explants (Fig. 2). This experiment has been repeated four times and the increase in sulfated GAG accumulation by db-cAMP-treated explants averaged 1·17 ($\pm$0·09) times controls on the first day of culture; 1·41 ($\pm$0·06) times controls on the second day of culture; and 1·89 ($\pm$0·19) times controls on the third day of culture. Since there was

little, if any, difference in the DNA content of control (average DNA content being $0{\cdot}50 \pm 0{\cdot}06$ μg/explant) and db-cAMP-treated ($0{\cdot}52 \pm 0{\cdot}07$ μg DNA/explant) explants during any period of culture, the increase in sulfated GAG accumulation/μg DNA in the presence of db-cAMP is not a reflection of a decrease in the amount of extractable DNA. The stimulatory effect of db-cAMP on sulfated GAG accumulation is dose-dependent in that 2·0 mM db-cAMP has a 20–25% greater stimulatory effect on sulfated GAG accumulation than does 1·0 mM db-cAMP (Table 1), while 0·1 mM db-cAMP has no detectable effect (Table 1). Furthermore, the stimulatory effect of db-cAMP is potentiated by the addition of the phospho-diesterase inhibitor, theophylline along with db-cAMP (Table 1), although theophylline alone has little, if any, effect on sulfated GAG accumulation (Table 1). As shown in Table 1, the stimulatory effect is not limited to the dibutyryl derivative of cyclic AMP, since 8-OH-cAMP also elicits a striking stimulation of sulfated GAG accumulation. The stimulation does, however, appear to be limited to cyclic AMP derivatives since neither 1·0 mM db-cGMP, nor 1·0 mM 5′-AMP have any effect on sulfated GAG accumulation (Table 1).

Overall, these results indicate that cyclic AMP derivatives, concomitant with inhibiting the AER-directed morphogenesis of subridge mesenchymal cells, elicit precocious chondrogenic differentiation of the cells. In order to confirm the latter biochemical observation, cartilage differentiation in control and cAMP-treated explants was examined histologically and histochemically. Sections of control and db-cAMP-treated explants stained with Alcian blue, pH 1·0, to histochemically examine the accumulation of cartilage matrix during the first 3 days of culture are shown in Fig. 3. As previously described (Kosher *et al.* 1979*a*), 1-day control explants are comprised entirely of unspecialized mesenchymal cells exhibiting a complete absence of Alcian blue-positive extracellular matrix (Fig. 3*a*). In contrast, Alcian-blue-positive material is already detectable in the proximal regions of 1-day db-cAMP-treated explants (Fig. 3*d*). During the subsequent 2 days of culture (when db-cAMP-treated explants have ceased undergoing AER-directed morphogenesis) progressively more distal tissue of the db-cAMP-treated explants acquires an Alcian-blue-positive extracellular matrix (Fig. 3*e*, *f*). In fact, by the third day of culture, virtually the entire explant is Alcian-blue positive, i.e. Alcian-blue-positive extracellular matrix extends distally up to the AER in db-cAMP-treated explants (Fig. 3*f*). In contrast, in control explants an Alcian-blue-positive extracellular matrix is only seen in these proximal regions of the explant that have become located greater than 0·4–0·5 mm from the AER, while the distal tissue within 0·4–0·5 mm of the AER remains Alcian-blue negative (Figs. 3*b*, *c*). These results thus confirm that in the presence of cAMP derivatives, cells within 0·4–0·5 mm of the AER, i.e. cells that would normally remain undifferentiated, undergo precocious chondrogenic differentiation.

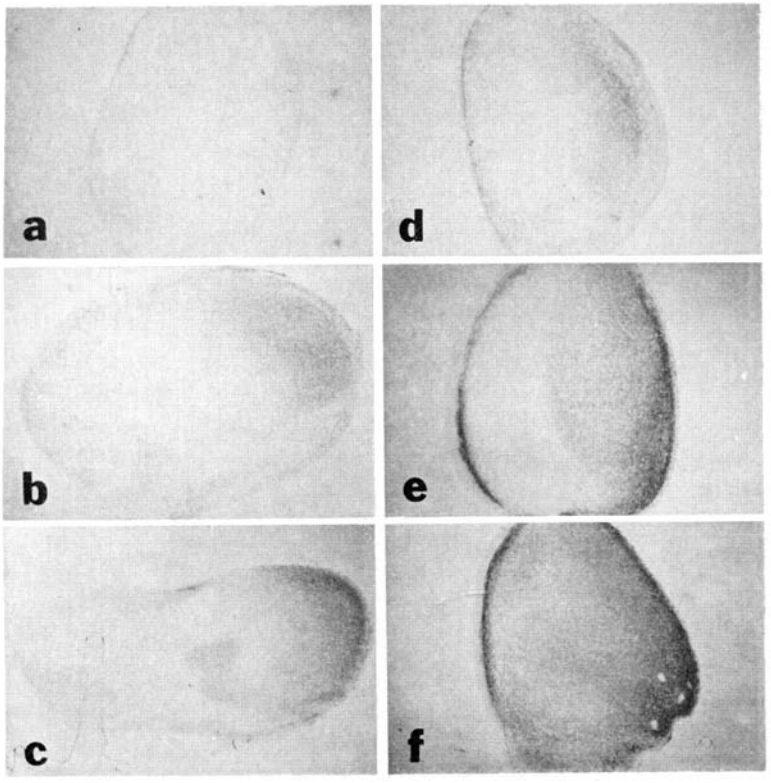

Fig. 3. (*a*) A section through a 1-day control explant stained with Alcian blue, pH 1·0. There is a complete absence of Alcian-blue-positive material throughout the explant. × 125.

(*b*)–(*c*) Alcian-blue (pH 1·0)-stained sections through 2-day (*b*) and 3-day (*c*) control explants. The proximal cells of the explants have acquired an Alcian-blue-positive extracellular matrix, while the distal cells of the explants that are still within 0·4–0·5 mm of the AER continue to exhibit an absence of Alcian-blue staining. × 125.

(*d*) An Alcian-blue (pH 1·0)-stained section through a 1-day db-cAMP and theophylline-treated explant. Note that in contrast to 1-day control explants (Fig. 3*a*) cells in the proximal region of the treated explants have acquired an Alcian-blue-positive extracellular matrix. × 125.

(*e*)–(*f*) Alcian-blue (pH 1·0)-stained sections through 2-day (*e*) and 3-day (*f*) db-cAMP and theophylline-treated explants. Note that during the period of culture progressively more distal tissue of the treated explants acquires an Alcian-blue-positive extracellular matrix. By the third day of culture (*f*), virtually the entire explant is Alcian-blue-positive. × 125.

DISCUSSION

The apical ectodermal ridge (AER) appears to be required for the outgrowth and formation of distal limb structures by the mesenchymal cells that constitute the bulk of the embryonic chick limb bud. Recent studies indicate that one of the major functions of the AER is to maintain mesenchymal cells directly subjacent to it (i.e. cells extending 0·4–0·5 mm from the AER), in a labile,

undifferentiated condition, and that when mesenchymal cells are freed from the AER's influence either artificially or as a result of normal polarized proximal to distal outgrowth, they are freed to commence cytodifferentiation (Kosher *et al.* 1979*a*; see also Stark & Searls, 1973; Summerbell *et al.* 1973). On the basis of the observation that a variety of agents that elevate cyclic AMP levels stimulate the already precocious chondrogenic differentiation that the subridge mesoderm undergoes in organ culture in the absence of the AER, we have suggested that when limb mesenchymal cells are removed from the influence of the AER, their cyclic AMP content increases thus triggering chondrogenic differentiation (Kosher *et al.* 1979*b*). The AER via molecule(s) produced by it may, therefore, exert its negative effect on the cytodifferentiation of subjacent mesenchymal cells by preventing either directly or indirectly this elevation in cyclic AMP content from occurring (Kosher *et al.* 1979*b*). In the present investigation, we have obtained evidence supporting this hypothesis by demonstrating that a variety of agents that should elevate cyclic AMP levels inhibit the morphogenesis of subridge mesenchymal cells cultured in the presence of the AER and concomitantly overcome the negative effect of the AER on cytodifferentiation. More specifically, in the presence of cyclic AMP derivatives, subridge mesenchymal cells fail to undergo the AER-directed polarized proximal to distal outgrowth and contour changes characteristic of control explants. Furthermore, in the presence of cyclic AMP derivatives, mesenchymal cells within 0·4–0·5 mm of the AER, i.e. cells that would normally be maintained undifferentiated, undergo precocious chondrogenic differentiation.

It is also of interest that in the presence of cyclic AMP derivatives the formation of cartilage by subridge mesenchymal cells that have ceased undergoing morphogenesis proceeds in a proximal to distal direction. The first cells of the cAMP-treated explants to undergo precocious chondrogenic differentiation are those proximal cells located farthest from the AER. Subsequently, cells located progressively closer to the AER undergo chondrogenic differentiation. This observation is consistent with the possibility that a molecule(s) produced by AER that is maintaining subjacent mesenchymal cells undifferentiated is distributed in a gradient-like fashion among the subjacent mesenchymal cells. That is, the concentration of the putative inhibitory molecule(s) may be greatest in the region immediately subjacent to the AER, and when cells as a result of polarized outgrowth become located progressively further from the AER, they have progressively less access to the inhibitory molecule(s) produced by the AER. If this were the case, one would expect, as we observe, that the negative effect of the AER would be progressively more difficult to overcome, the closer the cells are to the AER.

Although the results of the present study indicate that elevating the cyclic AMP content of the subridge mesenchymal cells enables the cells to overcome the negative influences on cytodifferentiation and the positive influences on morphogenesis being imposed upon them by the AER, it might be argued that

the observed effects may also be partially due to an impairment of the functioning of the AER. Although further experiments will be necessary to conclusively eliminate the latter possibility, several observations we have made argue against it. First of all, cyclic AMP derivatives have no noticeable effect on the gross morphology of the AER. The AER of cyclic AMP-treated explants remains thickened and similar in appearance to the AER of control explants. It does not flatten and undergo the degenerative changes characteristic of AER's that have lost their influence on subjacent mesenchymal cells (Kosher *et al.* 1979*a*; Rubin & Saunders, 1972; Zwilling, 1956*b*). That the primary effect of cyclic AMP derivatives is upon the mesenchymal cells is also suggested by our previous observation that cyclic AMP derivatives stimulate the already precocious chondrogenic differentiation that subridge mesenchymal cells undergo in the absence of the AER (Kosher *et al.* 1979*b*). Finally, in this regard, it is of interest that in the present experiments precocious chondrogenic differentiation by cAMP-treated subridge mesoderm explants in the presence of the AER progressively proceeds in a proximal to distal direction. This suggests that in the presence of cyclic AMP derivatives, the AER is still functioning and its effect is most intense upon cells immediately subjacent to it, since in cAMP-treated subridge mesoderm explants cultured in the absence of a functioning AER, an Alcian-blue-positive extracellular matrix is detectable throughout the entire explant by the end of the first day of culture (Kosher *et al.* 1979*b*). All of these observations thus suggest that in the present study cyclic AMP derivatives are preventing the mesenchymal cells from responding to the influence of the AER. It is possible, however, that, when in the presence of cyclic AMP derivatives subridge mesenchymal cell initiate cytodifferentiation, that the AER might then cease functioning since some evidence indicates that in order for the AER to exert its influence on limb mesodermal cells, an influence exerted on it by the mesodermal cells is required (see, for example, Saunders & Gasseling, 1968; Zwilling, 1961).

When the subridge mesenchymal cells are removed from the AER's influence either artificially (Kosher *et al.* 1979*a*, *b*) or as a result of polarized proximal to distal outgrowth in organ culture (Kosher *et al.* 1979*a*) and *in vivo* (Thorogood & Hinchliffe, 1975), they initially undergo condensation (i.e. become closely packed and apposed to one another) prior to becoming separated from one another by an Alcian-blue-positive cartilage matrix. A similar widespread cellular condensation also precedes overt cartilage formation by limb mesenchymal cells in high-density monolayer culture (Lewis, Pratt, Pennypacker & Hassell, 1978). However, we have not detected a widespread cellular condensation preceding overt cartilage formation in cyclic AMP-treated explants (Kosher *et al.* 1979*b*). This suggests the possibility that elevating the cyclic AMP content of the subridge mesenchymal cells precludes the necessity of cells passing through a condensation phase prior to overt cartilage formation. If this is the case, it further suggests that in the absence of exogenous cyclic

AMP, the intimate association between adjacent cell surfaces and the resultant cellular interaction that occurs during cellular condensation may result in the elevated cyclic AMP levels that are required to trigger chondrogenic differentiation. Obviously, however, our failure to detect widespread cellular condensation in the presence of cAMP derivatives does not necessarily mean that such a condensation does not occur. It is conceivable, for example, that in the presence of cAMP derivatives the mesenchymal cells pass through the cellular condensation phase so rapidly that we just missed detecting the process.

In summary, on the basis of all of the above considerations we propose the following skeleton model to serve as the basis for future experimentation. The AER synthesizes and secretes a molecule(s), the function of which is to maintain subjacent mesenchymal cells in an actively outgrowing, labile, undifferentiated condition. The 'inhibitory' molecule(s) produced by the AER becomes distributed in a gradient-like fashion among subjacent mesenchymal cells; the concentration of the 'inhibitory' molecule is greatest in the region immediately subjacent to the AER, and when cells as a result of polarized proximal to distal outgrowth become located progressively further from the AER, they have progressively less access to the 'inhibitory' molecule(s) produced by the AER. When cells as a result of continuing polarized outgrowth become located sufficiently far from the AER so that they no longer have access to the AERs 'inhibitory' molecule, the cells undergo condensation, i.e. become closely packed and apposed to one another. The resulting interaction between molecules on adjacent cell surfaces results in an elevation in the cyclic AMP content of the cells. As a result of the elevation in cyclic AMP content, chondrogenic differentiation is triggered. Alternately, the cyclic AMP content of the cells may increase when the cells no longer have access to the AER's inhibitory influence, but prior to cellular condensation. In either case, one of the virtues of the above model is that it provides a link between two critical aspects of limb development that have heretofore most frequently been studied as separate and distinct phenomenon, i.e. the effect of the AER on the outgrowth and formation of distal limb structures by limb mesodermal cells and the mechanism of cytodifferentiation. The major virtue of the model, however, is that virtually all aspects of it can be directly tested experimentally.

Finally, it should be noted that our model in its present form does not attempt to explain the orderly laying down of the various skeletal elements of the limb in their correct sequence along the proximo-distal axis. A potential explanation for this latter phenomenon has been suggested in the 'progress zone' model of Summerbell *et al.* (1973).

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REFERENCES

ABRAHAM, G. N., SCALETTA, C. & VAUGHN, J. H. (1972). Modified diphenylamine reaction for increased sensitivity. *Analyt. Biochem.* **49**, 547–549.

GOETINCK, P. F. (1966). Genetic aspects of skin and limb development. In *Current Topics in Developmental Biology* (ed. A. O. Monroy & A. A. Moscona), pp. 253–283. New York: Academic Press.

HAMBURGER, F. & HAMILTON, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morph.* **88**, 49–92.

HILFER, S. R., SEARLS, R. L. & FONTE, V. (1973). An ultrastructural study of early myogenesis in the chick wing bud. *Devl Biol.* **30**, 374–391.

JANNERS, M. Y. & SEARLS, R. L. (1970). Changes in rate of cellular proliferation during the differentiation of cartilage and muscle in the mesenchyme of the embryonic chick wing. *Devl Biol.* **23**, 136–165.

KOSHER, R. A. (1976). Inhibition of 'spontaneous', notochord-induced, and collagen-induced *in vitro* somite chondrogenesis by cyclic AMP derivatives and theophylline. *Devl Biol.* **53**, 265–276.

KOSHER, R. A. & LASH, J. W. (1975). Notochordal stimulation of *in vitro* somite chondrogenesis before and after enzymatic removal of perinotochordal materials. *Devl Biol.* **42**, 365–378.

KOSHER, R. A., SAVAGE, M. P. & CHAN, S.-C. (1979*a*). *In vitro* studies on the morphogenesis and differentiation of the mesoderm subjacent to the apical ectodermal ridge of the embryonic chick limb bud. *J. Embryol. exp. Morph.* **50**, 75–97.

KOSHER, R. A., SAVAGE, M. P. & CHAN, S.-C. (1979*b*). Cyclic AMP derivatives stimulate the chondrogenic differentiation of the mesoderm subjacent to the apical ectodermal ridge of the chick limb bud. *J. exp. Zool.* **209**, 221–228.

LEWIS, C. A., PRATT, R. M., PENNYPACKER, J. P. & HASSELL, J. R. (1978). Inhibition of limb chondrogenesis *in vitro* by vitamin A: alterations in cell surface characteristics. *Devl Biol.* **64**, 31–47.

LINSENMAYER, T. F., TOOLE, B. P. & TRELSTAD, R. L. (1973). Temporal and spatial transitions in collagen types during embryonic chick limb development. *Devl Biol.* **35**, 232–239.

RICHARDS, G. M. (1974). Modifications of the diphenylamine reaction giving increased sensitivity and simplicity in the estimation of DNA. *Analyt. Biochem.* **57**, 369–376.

RUBIN, L. & SAUNDERS, J. W. (1972). Ectodermal–mesodermal interactions in the growth of limb buds in the chick embryo: constancy and temporal limits of the ectodermal induction. *Devl Biol.* **28**, 94–112.

SAUNDERS, J. W. (1948). The proximo-distal sequence of origin of the parts of the chick wing and the role of the ectoderm. *J. exp. Zool.* **108**, 363–403.

SAUNDERS, J. W. & GASSELING, M. T. (1968). Ectodermal–mesenchymal interactions in the origin of limb symmetry. In *Epithelial–Mesenchymal Interactions* (ed. R. Fleischmajer and R. E. Billingham), pp. 78–97. Baltimore: Williams and Wilkins.

SAUNDERS, J. W., GASSELING, M. T. & ERRICK, J. E. (1976). Inductive activity and enduring cellular constitution of a supernumerary ectodermal ridge grafted to the limb bud of the chick embryo. *Devl Biol.* **50**, 16–25.

SEARLS, R. L. (1975*a*). An autoradiographic study of the uptake of S-35 sulfate during the differentiation of limb bud cartilage. *Devl Biol.* **11**, 155–168.

SEARLS, R. L. (1965*b*). Isolation of mucopolysaccharide from the pre-cartilaginous chick limb bud. *Proc. Soc. exp. Biol. Med.* **118**, 1172–1176.

SEARLS, R. L. (1973). Newer knowledge of chondrogenesis. *Clin. Orthop.* **95**, 327–344.

SEARLS, R. L., HILFER, S. R. & MIROW, S. M. (1972). An ultrastructural study of early chondrogenesis in the chick wing bud. *Devl Biol.* **28**, 123–137.

STARK, R. J. & SEARLS, R. L. (1973). A description of chick wing development and a model of limb morphogenesis. *Devl Biol.* **33**, 138–153.

SUMMERBELL, D. (1974). A quantitative analysis of the effect of excision of the AER from the chick limb bud. *J. Embryol. exp. Morph.* **32**, 651–660.

SUMMERBELL, D., LEWIS, J. H. & WOLPERT, L. (1973). Positional information in chick limb morphogenesis. *Nature New Biol.* **244**, 492–496.

THOROGOOD, P. V. & HINCHLIFFE, J. R. (1975). An analysis of the condensation process during chondrogenesis in the embryonic chick hind limb. *J. Embryol. exp. Morph.* **33**, 581–606.

YAMADA, K. (1970). Dual staining of some sulfated mucopolysaccharides with Alcian blue (pH 1·0) and ruthenium red (pH 2·5). *Histochemie* **23**; 13–20.

ZWILLING, E. (1956*a*). Interaction between limb bud ectoderm and mesoderm in the chick embryo. II. Experimental limb duplication. *J. exp. Zool.* **132**, 173–187.

ZWILLING, E. (1956*b*). Interaction between limb bud ectoderm and mesoderm in the chick embryo. IV. Experiments with a wingless mutant. *J. exp. Zool.* **132**, 241–253.

ZWILLING, E. (1961). Limb morphogenesis. *Adv. Morph.* **1**, 301–330.

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