

## Epithelial enhancement of connective tissue differentiation in explanted somites

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### SUMMARY

This paper examines the differentiation of somites from stage-16 or -17 chick embryos cultured with or without notochord in explant cultures. Histological sections of the cultures were stained with a trichrome stain to identify the different kinds of connective tissues formed. Both anterior and posterior (epithelial) somites made muscle, cartilage and loose connective tissue in explant culture. The extent of cartilage differentiation was enhanced by the presence of the notochord, confirming earlier studies. The presence of 1 mM-dibutyryl cAMP in the culture medium increased the amount of muscle found in the explants but by histological criteria did not inhibit chondrogenesis, contrary to earlier reports. The addition of quail ectoderm to the explants stimulated loose connective tissue to form directly beneath it, suggesting for the first time a role of the ectoderm in dermatome differentiation. These results suggest that the epithelial somite has the capacity to differentiate into all three connective tissue types even before it has separated into sclerotome and dermamyotome. The relative amount of different connective tissue types can be influenced by environmental factors, such as adjacent epithelia like the notochord or ectoderm.

### INTRODUCTION

In chick embryos, epithelial somites are formed from the segmental plate mesoderm in an anterior to posterior fashion (Williams, 1910). The epithelial somite subsequently differentiates into three identifiable regions, each of which will give rise to a specific type of connective tissue. The cells in the ventral-medial region are presumptive sclerotome. They become mesenchymal and disperse towards the neural tube and notochord (Solursh, Fisher, Meier & Singley, 1979), finally becoming vertebral cartilage. The dorsal part of the somite, the dermatome, contributes to the formation of the dermis (Rabl, 1888). The myotome, which develops into skeletal muscle, forms between these two layers (Paterson, 1888). The means by which these three distinct connective tissues differentiate from an epithelial ball remain obscure.

The induction of cartilage in somites has been studied extensively. The ventral spinal cord and notochord appear to promote the formation of cartilage from somite tissue *in situ* (Holtzer & Detwiler, 1953; Watterson, Fowler & Fowler, 1954). A number of other epithelia also promote somite chondrogenesis in

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chorioallantoic membrane cultures (O'Hare, 1972a). In explant cultures, somites are able to undergo spontaneous myogenesis (Ellison, Ambrose & Easty, 1969b) and chondrogenesis (Ellison *et al.* 1969a), if placed in a suitable medium. However, the notochord (Lash, 1963) or components of its associated extracellular matrix (ECM) (Kosher, Lash & Minor, 1973; Kosher & Church, 1975; Kosher & Lash, 1975; Lash & Vasan, 1978) enhance cartilage differentiation *in vitro*. In these later studies, cartilage differentiation was monitored largely by [ $^{35}\text{S}$ ] sulphate incorporation into glycosaminoglycans or proteoglycans. Because a reduction in sulphate incorporation per unit DNA was observed if somites and notochord or somites and some ECM components were cultured in the presence of cyclic AMP derivatives (Kosher, 1976) or calcium ionophore (Kosher, 1978), it was suggested that the notochordal ECM acts by reducing endogenous levels of cyclic AMP (Kosher & Savage, 1979).

In this study, histological criteria are used to assess the type of differentiation that occurs in somite explant cultures. In this way the extent or relative number of cells that differentiated into a particular type of connective tissue, rather than particular synthetic activities which can be independent of cell number, can be compared among different culture conditions. The results confirm previous studies that while the differentiation of cartilage, muscle, and loose connective tissues can occur spontaneously whether or not the notochord is present, the extent of differentiation is enhanced by particular environments. Cartilage is enhanced by the notochord. Unexpectedly, treatment of cultures with dibutyryl cyclic AMP increased the relative amount of muscle found in the explant without having any noticeable effect on the extent of cartilage differentiation. In addition, loose connective tissue formation is greatly stimulated by the presence of embryonic ectoderm.

#### MATERIALS AND METHODS

##### *Preparation of cultures*

Stage-16 or -17 chick embryos (Hamburger & Hamilton, 1951) were removed from fertile White Leghorn eggs (Welp Corp., Bancroft, Iowa) with filter paper rings as described previously (Lash, 1967). Embryos were placed in sterile Simms' buffered salt solution (SBSS, Simms & Sanders, 1942) and trunk regions were isolated by cutting just anterior to the wing bud, posterior to the last somite formed, and just lateral to the somites. A few of these were fixed in Bouin's, stained with Milligan's trichrome stain and cut in cross-section (Fig. 1). After four to five trunk regions were isolated, two were transferred to 1% trypsin in calcium- and magnesium-free saline G for 18 min at 4°C. The other trunk pieces were left on ice in SBSS. After enzyme treatment, the trunk sections were placed in horse serum mixed with SBSS in a ratio of 1:1 or 1:2 and put back on ice for 3–5 min before dissection. After removing the ectoderm and lateral plate

mesoderm from the somites with sharpened tungsten needles, the neural tube was teased away starting from the posterior end and advancing anteriorly. Using this procedure, it was possible to isolate the notochord with the somites still attached on either side without any other surrounding tissue. Notochord and somites were then left in medium on ice until all trunk regions had been dissected.

Somites isolated from each embryo were cut into sections for explant cultures, such that each piece contained three somites on either side of the central notochord. The notochord was removed before culturing for explants without notochord. The explants were divided into three groups: one group contained anterior somites in which the sclerotome and dermamyotome were easily recognized, another contained only the most posterior somites that were still undifferentiated epithelial balls, and a third group consisted of somites in intermediate stages of development. Cross sections through the anterior and posterior somites before isolation from dissected trunk regions are shown in Fig. 1. Explants were then cultured as described by Cheney & Lash (1981). Briefly, somites with or without notochord were placed on Nucleopore filters (0.4 to 0.8  $\mu$ m pore size) supported by a stainless steel grid. Enough culture medium (F<sub>12</sub> X, SBSS, and foetal calf serum in a ratio of 2:2:1 with penicillin and streptomycin) was added to just reach the edge of the explant, and the dish was then placed in a humidified 95 % air, 5 % CO<sub>2</sub> incubator at 37 °C. Medium was replaced each day and cultures were fixed after 3 or 6 days of culture. In some cases, cultures were fed with medium containing 1 mM db cAMP (N<sub>6</sub>, O<sub>2</sub>-dibutyryl adenosine 3',5' cyclic monophosphoric acid, sodium salt from Sigma Chemical Company) from either day 0 or day 1 of culture until fixation.

Trunk ectoderm was obtained from trunk regions removed from stage-16 or -17 Japanese quail embryos as described above. Isolated trunks were cut into anterior, middle, and posterior regions before incubating in 1 % trypsin for 8–10 min at 23 °C. Ectoderm was removed in equal parts SBSS and horse serum with tungsten needles and transferred to medium after dissection. Ectoderm from a particular region was added to somites and notochord from the same region. The ectoderm was added on day 1 of culture, and the explants were fixed either 2 or 5 days later. Quail ectoderm was used so that it could be distinguished cytologically from the chick somites by the presence of the distinctive quail nucleolar marker (Le Douarin, 1973).

### *Histology*

Explants attached to the filters were rinsed in Saline G and fixed in Kahle's fixative for 30 min. After a rinse in distilled water, the cultures were left in alcian blue (pH 1) overnight and destained with 0.1 N HCl. This enabled one to see the position and orientation of the notochord in the explant, so that the explant could be cut later in cross section through the notochord. Explants were then dehydrated in a series of ethanol and embedded in paraffin. Seven  $\mu$ m serial

sections were cut and stained with Milligan trichrome stain (Humason, 1972), Carazzi haematoxylin, or Schiff's reagent. With the trichrome stain, different connective tissue types were easily identified. Cartilage was a dark blue-green, muscle was pink with striations visible under high power. Loose connective tissue cells were red, surrounded by a characteristic highly dispersed alcian blue/fast green staining matrix, and red staining cells with no differentiated morphologies were classified as mesenchymal.

Serial cross sections of each explant were examined. In some cases, every 10th or 15th section was projected with a Leitz projector at 350 $\times$  and traced. The area of the entire section, muscle tissue, and cartilage tissue was then determined with a Talos digitizer interfaced with a Monroe programmable calculator. Generally, sections near the centre of each explant were found to be most representative of the different types of tissue formed and the amount of each tissue found in the entire explant. Such sections were photographed and are shown in the results. Direct comparisons are made only between explants prepared at one time because there was usually some variation from one trial to the next. However, the results within an experiment were consistent each time.

## RESULTS

### *Effect of notochord*

In the trunk region of a stage-16 or -17 chick embryo, there was a range of differentiation among the somites. The posterior somites were still epithelial in nature (Fig. 1A), while the somites from the anterior region had separated into sclerotome and dermamyotome, which can be seen clearly in cross section through an isolated trunk region (Fig. 1B). Even though the morphological appearance of the somites varied dramatically before culturing, the results obtained in explant cultures were similar for all three groups: anterior, middle, and posterior. Anterior somites cultured without notochord formed areas of both cartilage and muscle that can be seen histologically in cross section (Fig. 2A). Epithelial somites from the middle (Fig. 2B), and posterior region (Fig. 2C) also differentiated into cartilage and muscle. In addition, tissue with a mesenchymal appearance was found throughout explants of somites from any level. Somites from the three different regions were compared in six independent trials, and duplicate cultures within an experiment were compared in at least four of those trials.

The addition of notochord to somite cultures resulted in increased cell survival, suggested by the much larger size and healthier appearance of the explants (compare Fig. 2A, B and C with D, E and F). In addition, a higher percentage of explants survived with notochord (30/42, 71 %) than without notochord (16/35, 46 %). At day 3, somites from all regions of the trunk cultured with notochord contained extensive areas of cartilage, demonstrated by staining with alcian blue at pH 1 or fast green. Direct comparisons between duplicate cultures

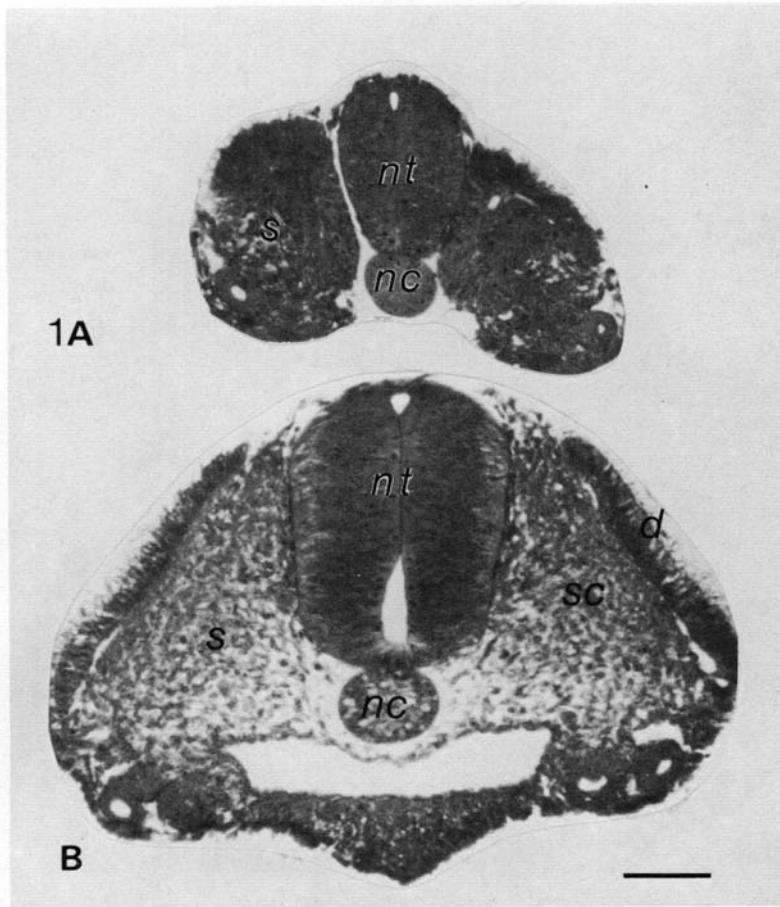


Fig. 1. Cross sections through the trunk region dissected from a stage 16 chick embryo and fixed in Bouins. Labelled s (somite), nt (neural tube) and nc (notochord). A) Cut through the posterior end of a trunk region. Somites are still epithelial in nature. B) Sectioned through the most anterior somites in the isolated trunk region. Sclerotome (sc) and dermamyotome (d) are already clearly separated. Bar = 100  $\mu$ m.

from all three embryonic areas with and without notochord were made for three different experiments. The notochord in each case enhanced survival of the somite mesoderm and the amount of cartilage tissue formed. Cartilage was typically found in the area surrounding the notochord (Fig. 2E, F, G, 3A and 4). Large muscle masses were also commonly seen in these cultures (Fig. 2D, E, F, G, 3A and 4). Occasionally, small areas resembling loose connective tissue were also observed in the explants (Fig. 3A and 4).

#### *Effect of dibutyryl cAMP (db cAMP)*

Treatment of somites cultured with notochord from either day 0 or day 1 of culture with db cAMP increased the relative amount of muscle in the explant,

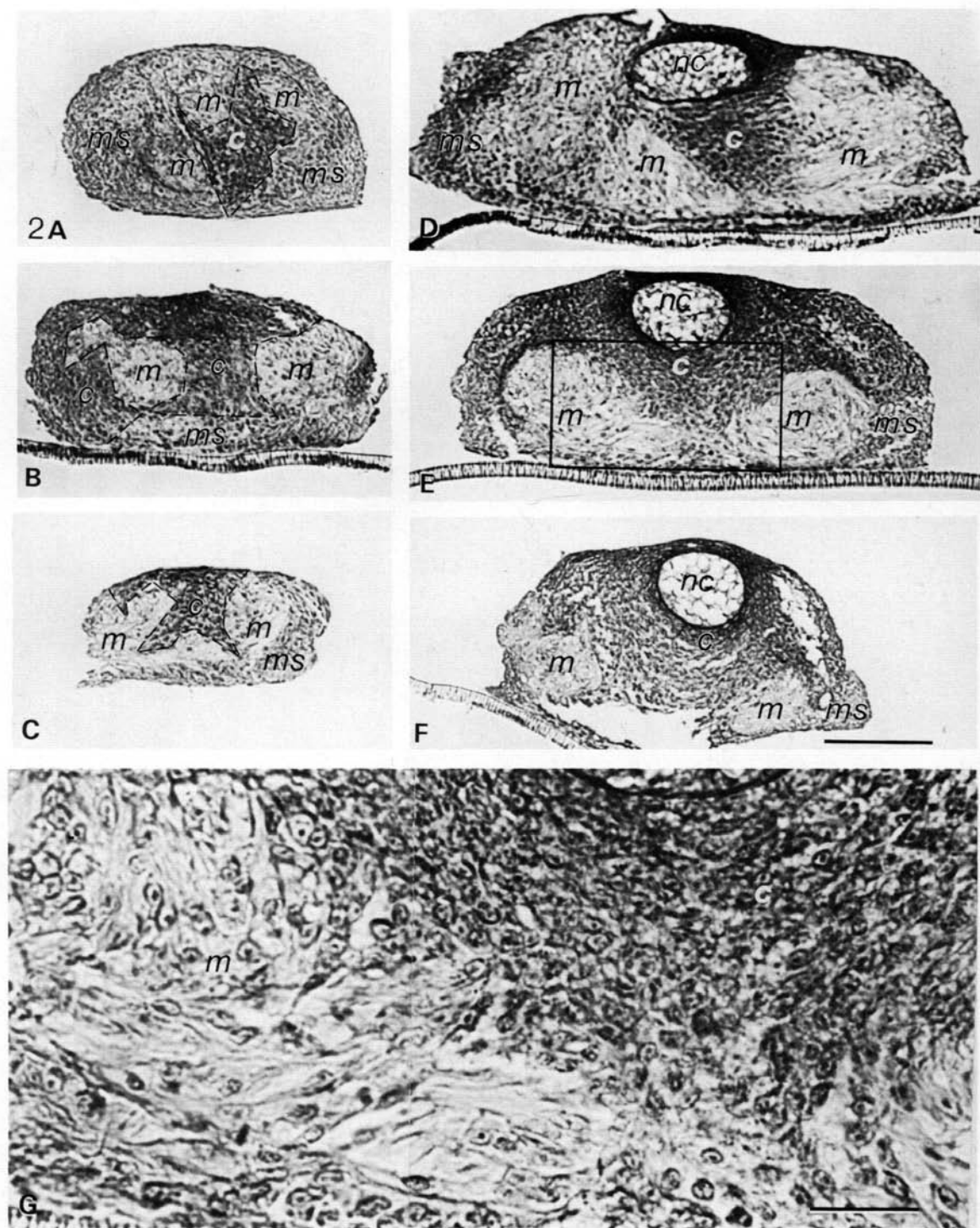


Fig. 2

Table 1. *Effect of db cAMP treatment on somite explants fixed on day 3*

	Control*†		1 mM-db cAMP day 1-3*†	
	muscle total area	cartilage total area	muscle total area	cartilage total area
Anterior	0.046	0.273	0.362	0.287
Middle	0.015	0.388	0.342	0.357
	0.142	0.374	0.391	0.290
	0.017	0.143	0.276	0.330
	0.249	0.430	0.357	0.322
Posterior	0.061	0.423	0.259	0.479
	0.229	0.515	0.238	0.422
			0.280	0.396
	$\bar{X} = 0.018$	$\bar{X} = 0.364$	$\bar{X} = 0.313$	$\bar{X} = 0.360$

\* Areas were measured for cartilage, muscle and the total section for four to six sections per explant. Ratios (area of specific tissue/area of whole section) were calculated for each section, and the ratio reported is a mean of all sections measured in the explant. Each value represents the ratio for a different explant.

† The ratio of cartilage per total area in treated explants was not significantly different from control explants when tested with the Mann-Whitney U Test. Amount of muscle per total area in treated cultures was significantly higher than in the untreated cultures (Mann-Whitney U Test,  $P < 0.001$ ).

while the amount of mesenchyme was decreased. This difference is demonstrated quantitatively in Table 1. The relative amount of cartilage in db cAMP treated cultures was not significantly different from control cultures (Table 1). For explants fixed on day 3, duplicate control and treated cultures from all three regions were compared histologically in four independent trials. Two experiments were analysed quantitatively, and the results are shown in Table 1. The effect of db cAMP treatment can be readily observed histologically by day 3 of culture by comparing posterior somites (which normally make small amounts of muscle) (Fig. 3A) with db cAMP treated posterior somites (Fig. 3B).

Fig. 2. Explants from stage-17 chick embryos cultured for 3 days, cut in cross section and stained with alcian blue and haematoxylin. Cartilage (white c) stains darkly, and is outlined by dashed lines (---) in A), B) and C). In D), E) and F) it is clearly seen as the darkly staining area surrounding the notochord. Muscle (m) is found in the lightest areas. Non-alcian blue staining tissue without visible striations is labelled as mesenchymal tissue (ms). A), B) and C) Somites grown without notochord. D), E) and F) Somites cultured with notochord. A) and D) Somites were from the anterior region (already differentiated into sclerotome and dermamyotome at the time of explanting). B) and E) Somites from the middle region (partially differentiated). C) and F) Posterior somites which were still epithelial when explanted. All cultures were set up and fixed on the same day. Notice the presence of both cartilage and muscle in all cultures in addition to some mesenchymal tissue. Bar = 100  $\mu$ m. G) High power magnification of the area outlined in E), containing cartilage (c) and muscle (m) tissue. Bar = 25  $\mu$ m.

The effect of db cAMP on muscle tissue formation was also dramatic in cultures fixed on day 6 in somites from all three regions. Control somites and notochord from the anterior region fixed on day 6 are shown in Fig. 3C. In all explants (four trials carried out with duplicates from each area in at least two of the trials) without db cAMP treatment there was very little muscle tissue seen on day 6. Instead there were large necrotic spaces and some undifferentiated tissue

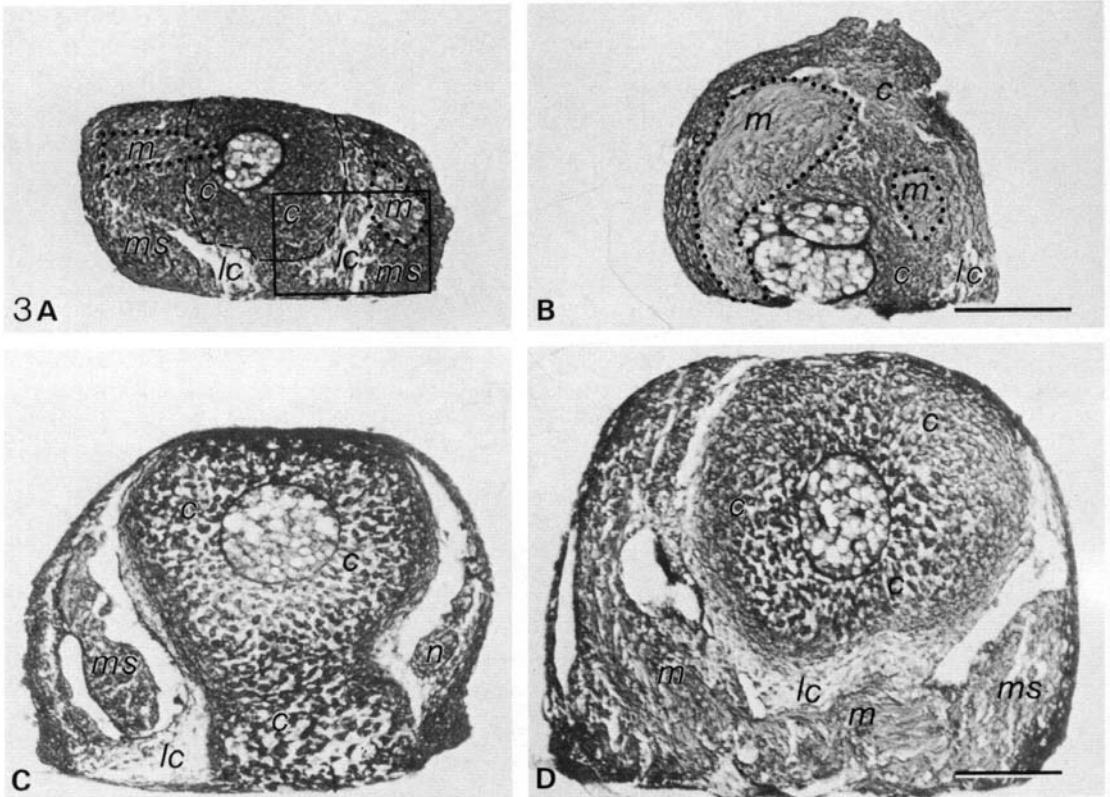


Fig. 3. Control (A, C) and db cAMP treated (B, D) explants of somite and notochord from stage-16 chick embryos stained with Milligan trichrome stain. A) and B) Somites and notochord from the posterior region in one experiment fixed on day 3. Muscle areas are labelled (m) and outlined by dotted lines (. . .). Cartilage (c), loose connective tissue (lc) and mesenchymal (ms) tissue areas are labelled. A) Control culture. Cartilage is shown by a dashed line (- - -). A small amount of muscle is also present in addition to mesenchymal tissue. Solid line outlines area shown in high-power magnification in Fig. 4. B) db cAMP treated culture. Note the increase in muscle area relative to total tissue. This culture has very little mesenchymal tissue. Bar = 100  $\mu$ m. C) and D) Somites and notochord from the anterior region cultured simultaneously and fixed on day 6. C) Control culture showing extensive cartilage (c) around the notochord, some loose connective tissue (lc), and some mesenchymal (ms) and necrotic tissue (n) in the area where muscle is seen on day 3. D) Culture treated with 1 mM-db cAMP from day 1 to day 6 labelled as in C). In addition to cartilage, loose connective tissue, and mesenchyme, much more muscle tissue is found. Bar = 100  $\mu$ m.



in approximately the same place where muscle and mesenchyme were found on day 3 (compare Fig. 3C with Fig. 2D). If treated from day 1 to day 6 of culture with 1 mM-db cAMP, the somites and notochord explants contained large muscle areas and fewer necrotic areas. Fig. 3D shows a db cAMP treated anterior explant of somites and notochord. Compare the amount of muscle tissue present in Fig. 3C and D. Duplicate explants were compared between control and db cAMP treated cultures in two independent trials.

#### *Effect of ectoderm*

In most explants fixed on day 6, there was loose connective tissue found (Figs. 3C and 3D), which was larger in extent than that seen at day 3 (Fig. 3A, high-power mag. Fig. 4). Even somites without notochord can form some loose connective tissue if fixed on day 6 (not shown). If quail ectoderm was grafted on day 1 to the top of an explant of somite plus notochord, very little difference was seen in cross section if fixed on day 3 (not shown). Typically, directly beneath the quail ectoderm, there was a small amount of chick tissue present that had more extensive intercellular spaces. By day 6, however, extensive areas of loose connective tissue had formed at this location (Fig. 5B, C and D) directly under the ectoderm. Figure 5C confirms the fact that the loose connective tissue is of chick origin underlying the quail ectoderm. Also, the cultures contained as little muscle as control cultures (Fig. 5A), but there was still considerable cartilage tissue present. Three independent trials were carried out, and in every instance where quail ectoderm could be positively identified, there was chick loose connective tissue associated with it.

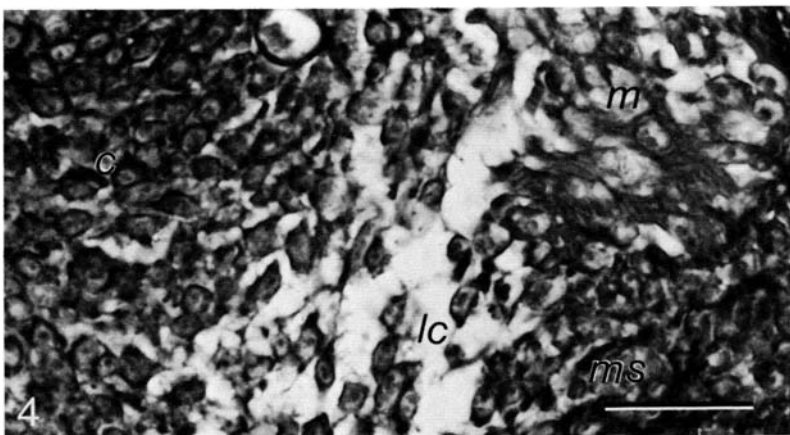


Fig. 4. High-power magnification of the boxed-in area in Fig. 3A. This is an explant from the posterior region fixed on day 3. In addition to the blue-green cartilage (c) and pink muscle (m) tissue, there is a small amount of loose connective tissue (lc) and red mesenchymal tissue (ms). Bar = 25  $\mu$ m.

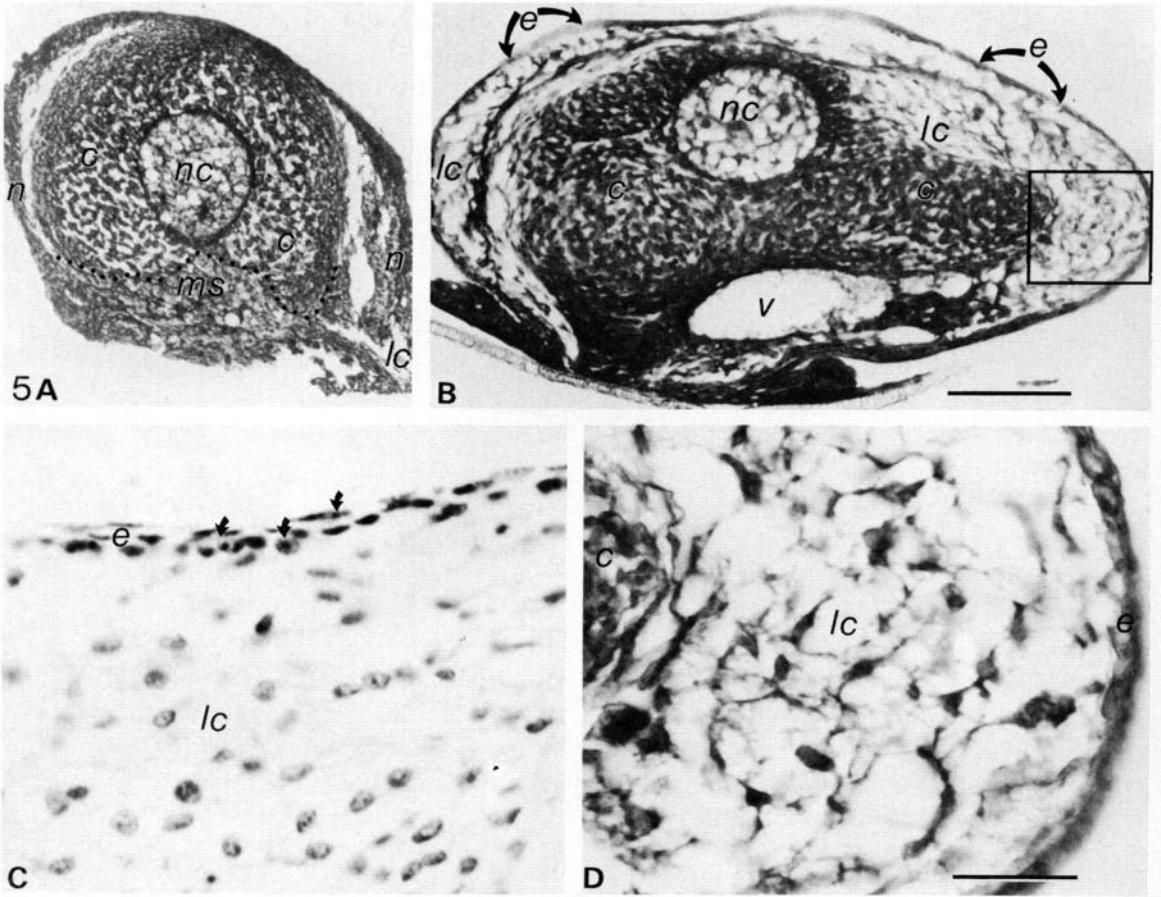


Fig. 5. A) Posterior somites and notochord fixed on day 6. There is extensive cartilage (c) around the notochord with necrotic tissue on the edges (n) and some mesenchymal tissue (ms) near the filter. B) Somites and notochord from the posterior region with quail ectoderm grafted onto the explant on day 1. Note the extent of the loose connective tissue under the ectoderm (e). Extensive cartilage (c) surrounds the notochord. Large interconnecting vesicular networks (v) were also often found in the loose connective tissue of these cultures. Both A) and B) were stained with Milligan's trichrome stain to see matrix detail. Bar = 100  $\mu$ m. C) and D) High-power magnification showing the quail ectoderm and chick connective tissue beneath it. Bar = 25  $\mu$ m. C) This explant was stained with Schiff's reagent to confirm that the connective tissue was of chick origin. Quail ectoderm can be identified by the distinctive nucleolar marker in the cells (arrows). D) Area outlined in B shows the extracellular matrix of the chick connective tissue found under the quail ectoderm.

#### DISCUSSION

We have confirmed the results of Ellison *et al.* (1969a and 1969b) that stage-16 or -17 chick embryo somites can form both cartilage and muscle spontaneously *in vitro*. In addition, some of the somite tissue differentiates into loose connective tissue, a result that had not been noted previously. Therefore, by stage 16/17 the

somites are already capable of differentiating *in vitro* into the three tissue types they would eventually have formed *in vivo*. It is not yet known whether these three cell types originate from a common precursor cell type or from different cell lineages already present in the epithelial somite. It would be important to know whether the cartilage and muscle came from the presumptive sclerotome and dermamyotome tissue, respectively. Although this is the most likely explanation, there is some uncertainty in this study because of the flattening of the tissue on the filter. Also, because explants were cultured for 3 days before fixation, we cannot rule out the possibility that the tissue reorganized during the culture period.

The relative and absolute amounts of cartilage, muscle, and loose connective tissue could be influenced by the culture conditions. The addition of notochord to the somites increased not only the amount of cartilage made as reported earlier by Lash (1963) but also increased the survival of the explants and reduced the amount of mesenchyme seen in the cultures. Earlier, Gordon & Lash (1974) noted that a major effect of the notochord *in vitro* is to promote cell survival. This study confirms their results and shows that the increase in incorporation of sulphate is due to an actual increase in the amount of cartilage tissue present in the somite explants.

The present study also shows that db cAMP treatment increases the amount of muscle in somite explant cultures. This effect is detectable quantitatively on day 3 and is qualitatively unequivocal by day 6. The effect of db cAMP on myogenesis in explant culture has not been previously reported. Although Kosher (1976) suggests that db cAMP treatment inhibits chondrogenesis of explanted somite cultures based on  $S^{35}$  incorporation per unit DNA, the present results raise the possibility that the inhibition he reported was only an apparent inhibition, caused by an increased proportion of muscle tissue. An effect of db cAMP on myogenesis is not entirely unexpected. Although one effect of db cAMP on myoblasts in cell culture is to prevent fusion (Zalin, 1973), this may be due to a reduced growth rate (Epstein, Jiminez de Asua & Rozengurt, 1975). Db cAMP actually promotes fusion in culture in the presence of suboptimal  $Ca^{++}$  concentrations (Aw, Holt & Simons, 1974). In addition, increased endogenous levels of cyclic AMP are correlated with myoblast fusion (Zalin & Montague, 1974). While db cAMP treatment increased the amount of muscle and decreased the amount of mesenchyme found in the explants, the results from the day 6 experiments suggest that db cAMP acts by increasing the viability of muscle in this medium.

The ability of the ectoderm to promote loose connective tissue formation by explanted somites has not been described previously. Earlier related studies focused on the ability of some epithelia to promote chondrogenesis (Seno & Büyükozer, 1958; O'Hare, 1972a) and did not utilize the quail marker so that the source of the connective tissue could be ascertained with certainty. The fact that a small amount of loose connective tissue is associated with the ectoderm on day

3 suggests that the extended culture period allows proliferation of such tissue. It is not known whether loose connective tissue formation is due to selective survival of presumptive dermatome cells or whether the ectoderm directs the differentiation of underlying mesenchyme by instructive induction. In either case, these results demonstrate that the ectoderm favors the formation of loose connective tissue. It is important to consider that the ectoderm might provide such a stimulus *in situ*, as well. A similar effect of ectoderm on chick somites cultured *in vivo* has been noted by Christ (1969). It is possible that generally ectoderm promotes loose connective tissue formation from mesenchyme, since it has a similar effect on limb mesenchyme in high density cell cultures (Solursh, Singley & Reiter, 1981). In this case also, the notochordal epithelium did not have such an effect.

In conclusion, we have shown that stage 16/17 somites (both epithelial somites and more advanced stages) are capable of forming a variety of tissues in explant culture. The amount of any particular tissue type can be influenced by specific environmental conditions imposed upon the cultures. These results are consistent with the idea that the mesenchymal cells are already committed to form these connective tissues and various permissive conditions allow proliferation of the determined cell types. The alternative hypothesis that the uncommitted somite tissue is directed to form a certain tissue by culture conditions cannot yet be eliminated. In either case, the results emphasize the importance of adjacent epithelia in the differentiation of connective tissues. The notochord promotes the extensive differentiation of cartilage, while the ectoderm promotes loose connective tissue formation. The origin of the precursor cells for each of the three connective tissue cell types is still unknown.

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