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Cell Biology

De novo induction of a gene product during heterologous epithelial–mesenchymal interactions *in vitro*

(induction of collagen types/*in vitro* chondrogenesis)

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ABSTRACT **Mesenchymal specification of epithelial cytodifferentiation and morphogenesis has been considered to be a general feature of various epithelial–mesenchymal interacting systems (e.g., salivary gland, mammary gland, feather, hair, and tooth morphogenesis). In contrast, we have demonstrated that a mesenchyme can be induced by a heterologous epithelium to synthesize in quantity a specific gene product(s) unorthodox to the organ from which the mesenchyme was taken. Stage 22–23 avian limb bud epithelium induced 17-day embryonic mouse tooth mesenchyme to differentiate into cartilage. Peptide analysis (cyanogen bromide cleavage after purification of extracted collagen chains) demonstrated that heterologous tissue recombinations produced type II collagen $[\alpha(II)]_3$ (i.e., cartilage-type) in addition to type I collagen $[\alpha(I)]_2\alpha_2$. Intact or reconstituted mouse molar tooth organs synthesized type I collagen and type I trimer $[\alpha(I)]_3$ collagen. Immunohistochemical criteria using anti-type II collagen antibodies identified type II collagen in cartilage-like matrix within the mesenchymal component of heterologous tissue recombinants. Cartilage has never been described during *in vivo* or *in vitro* tooth tissue differentiation or associated with the pathology of dental papilla mesenchyme. These results support the hypothesis that epithelial–mesenchymal interactions during embryonic development can selectively induce *de novo* synthesis of unique gene products.**

Many studies of embryonic induction have been concerned with the expression of a genome that has been preselected before the experimental observations began (see refs. 1–3 for review). These studies have been useful in providing information regarding regulation of translation of previously activated structural genes but have not described how preferential selection of specific genes might occur in complex developing organ systems. The nature of the instructions for such developmental programs remains obscure, although empirical information is available regarding several developmental programs (see selected refs. 4–9 for review).

The classic experiments of Spemann (6) provided convincing evidence regarding the importance of heterotypic tissue interactions during morphogenesis; tissues were induced to differentiate into specialized structures that they would never form during normal development *in situ*. More recently, examples have been provided in which mesenchyme produced permissive or rate-limiting factors that mediated epithelial cytodifferentiation and morphogenesis (4, 5). Grobstein (2, 10, 11) emphasized the significance of *reciprocal* epithelial–mesenchymal interactions associated with epidermal organ systems. Epithelium or mesenchyme or both have been suggested to serve (*i*) to regulate previously determined gene expression without nascent gene activation and transcription, or (*ii*) to direct mRNA synthesis toward *de novo* production of specific gene products. In order to assess induction during epithelial–mesenchymal interactions, we designed studies to evaluate tissue recombinations between tissues of disparate organ systems and across vertebrate species. The present paper demonstrates *de novo* induction of type II collagen synthesis in mouse tooth mesenchyme as a direct consequence of recombination with avian epithelium *in vitro*, reopening an inquiry into mechanisms by which intercellular communication mediates complex developmental processes.

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MATERIALS AND METHODS

Preparation of Cultures. Cultures of avian limb epithelium and mesenchyme were obtained from either Japanese quail (*Coturnix xoturnix japonica*) or White Leghorn chicken embryos. Limb buds from stages 22 and 23 (12) were removed and separated into epithelium and mesenchyme. Molar tooth organs (cap stage) were obtained from 17-day embryonic CD-Swiss mice and dissociated into enamel organ epithelium and dental papilla mesenchyme (13). Limb buds and tooth organs were placed in Hanks' solution (GIBCO, HBSS K-12) at 4°. Dissociation was accomplished with 1% trypsin (Difco, 1:250) in Ca^{2+}- and Mg^{2+}-free Tyrode's solution (pH 7.4) at 4° for 75 min. Trypsin was inactivated with 50% fetal calf serum in Hanks' solution. Tissues were cultured in the following scheme: (*i*) intact limb buds, (*ii*) intact tooth organs, (*iii*) avian mesenchyme, (*iv*) avian epithelium, (*v*) mammalian mesenchyme, (*vi*) mammalian epithelium, (*vii*) reconstituted tooth epithelium and mesenchyme, and (*viii*) avian epithelium and mouse tooth mesenchyme recombinations. Explants were cultured on Millipore filters on the chorioallantoic membrane of 7.5-day chicken embryos for periods up to 7 days (14) or were cultured *in vitro* by using a modified Trowell procedure (Millipore filters, 0.45 μm pore and 25 μm thick) in plastic petri dishes (100 × 15 mm, Lux Scientific Co.) containing Eagle's minimal essential medium (GIBCO, F-11) supplemented with 10% fetal calf serum (GIBCO), sodium bicarbonate (1.35 mg/ml), glycine (50 μg/ml), and gentamycin (50 μg/ml, Schering Company). These conditions were found to be permissive for tooth morphogenesis as assayed by the expression of dentine and enamel matrix (13). After 5 days *in vitro* (unless specified) in a humidified incubator at 37° and 5% CO_2/95% air, explants from each group were examined by using routine histologic and immunochemical procedures or were processed for biochemical analyses (Fig. 1). Several hundred explants of each group were required for each experiment. All experiments were replicated three times.

Histological and Immunohistochemical Studies. Explants for histological observations were fixed in 10% neutral formalin for 24 hr at 4°, embedded in paraffin, sectioned, and then

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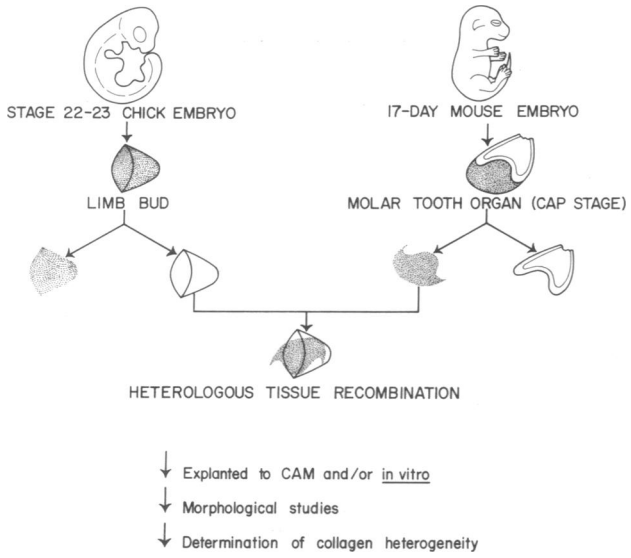

FIG. 1. General experimental scheme to assess the effects of heterologous epithelial–mesenchymal interactions on embryonic induction. CAM, chorioallantoic membrane.

stained with hematoxylin and eosin. To localize type I procollagen and type II collagen, we used indirect immunofluorescence microscopy (15) with specific antibodies against these collagens. Explants from each experimental group were kept in 30% sucrose for 24 hr at 4°, and then frozen sections (4–6 μm thick) were cut and treated with 2% testicular hyaluronidase (15) for 30 min before incubation with antisera. Antisera specificity was evaluated by using frozen frontal sections of newborn mouse heads containing regions of intramembranous and endochondral ossification, Meckel's cartilage, tooth development, and related connective tissues. Anti-type II collagen serum was routinely absorbed with skin type I collagen before use. Control experiments indicated that nonspecific association of label was negligible.

Purification of Radioactive Collagen. Selected experimental groups, either before or after 5 days in culture, were cultured in fresh medium (as described above) supplemented with β-aminopropionitrile (50 μg/ml; Aldrich) and [2,3-^{3}H]proline (0.1 mCi/ml; specific activity, 24.5 Ci/mmol). Proline concentrations were adjusted to 0.11 mM by the addition of L-proline. After 24 hr of incubation, subcultured explants and their medium were separately combined with 3 mg of unlabeled collagen carrier (type I collagen from skin). Samples were homogenized and, after the addition of 1 mg of L-proline, were dialyzed against 0.5 M acetic acid for 48 hr. Extractions and purification procedures were done at 4°. Samples (10–50%) of the retentate were treated with cold 10% trichloroacetic acid to be used for DNA determinations (16), and total collagen was determined by using protease-free collagenase (Advanced Biofractures, form III) (17). In the presence of *N*-ethylmaleimide (2.5 μM), non-collagen-degrading protease activity was not detected. The remainder of each sample was centrifuged, and the precipitate was extracted twice with 0.5 M acetic acid for 24 hr. We found that 95% of the [^{3}H]proline-labeled collagen was recovered in the extraction procedures. Collagen was precipitated from combined extracts by the addition of NaCl to a final concentration of 0.9 M. This precipitation procedure was repeated twice.

Differential Salt Precipitation to Isolate Different Types of Collagen. Isotopically labeled samples were mixed with type I and type II collagen carriers. Samples were dialyzed first against 1.0 M NaCl/0.05 M Tris·HCl, pH 7.5, at 4° and then against 2.6 M NaCl/0.05 M Tris·HCl, pH 8.0, at 4° (18). The resulting precipitate was collected by centrifugation. The collagen remaining in the supernatant was precipitated by dialysis against 0.01 M Na_2HPO_4. Each precipitate was then dissolved and dialyzed against the starting buffer for subsequent CM-cellulose chromatography.

Analysis of Collagen Types after Cyanogen Bromide Cleavage. Radioactively labeled collagen and collagen carriers were eluted from CM-cellulose columns and then mixed with 4–7 mg of purified collagen chains. Samples were then dissolved in 2 ml of 70% formic acid, gassed with nitrogen after the addition of 30 mg CNBr, and incubated at 30° for 4 hr (19); reaction was terminated by the addition of water. Cleavage products were lyophilized three times.

CM-Cellulose/Chromatography to Separate Various Collagen α Chains and CNBr Peptides. Native collagen molecules were denatured by heating at 50° for 10 min and then separated on a 0.6 × 15 cm column of CM-cellulose (Whatman, CM-52). Columns were washed with 0.04 M sodium acetate/4 M urea, pH 4.8, and eluted with a salt concentration gradient from 0 to 72 mM with 50 ml each of starting buffer and limit buffer run at 43°. Fractions were collected as reported (19, 20). The CNBr peptides were chromatographed on an identical column to separate individual α-chains. For elution of CNBr peptides, columns were washed with starting buffer (0.02 M sodium citrate/0.02 M NaCl) and then developed with a salt concentration gradient with 200 ml each of starting and limit buffer (21).

RESULTS

Avian Epithelium "Induced" Mammalian Tooth Mesenchyme to Express Chondrogenesis. Avian epithelium induced tooth mesenchyme to differentiate into cartilage-like tissue (Fig. 2). Cultures of heterotypic tissue recombinations resulted in a modification of the dental mesenchyme phenotype and the induction of chondrogenesis. Isolated tooth mesenchyme did not form dentine, but rather appeared as a fibroblast outgrowth. Recombined dental tissue formed molariform structures and expressed normal tooth characteristics for dentinogenesis and amelogenesis (3, 7, 13, 14). Isolated chicken epithelium formed a sheet of keratin in culture. Avian mesenchyme always expressed cartilage formation in culture, indicating that limb mesenchyme had been determined prior to the initiation of these experiments.

Antibodies to type I procollagen and type II collagen were specific. Unequivocally, both types of collagen antigens were identified over the mouse mesenchyme in heterologous tissue recombinants. Chondrogenic cells were stained with antibody against type II collagen (Fig. 2*D*). Tooth organs, reconstituted tooth tissue recombinants, isolated tooth mesenchyme, and isolated chicken limb epithelium did not stain with type II antibodies.

Characterization of Collagens Synthesized in Cultures. Intact 17-day mouse tooth organs synthesized low levels of type I collagen and $[a(I)]_3$ (Table 1). After 5 days in culture, total collagen synthesis was amplified 20 times. Isolated mouse mesenchyme, cultured for comparable periods of time, showed no increase in collagen synthesis when expressed on a per tissue basis.

No detectable collagen was synthesized by chicken epithelium. Heterologous recombinants between chicken and mouse tissues resulted in a highly significant increase in [^{3}H]proline incorporation into collagen.

***De Novo* Synthesis of Type II Collagen Resulting from Epithelial–Mesenchymal Interactions.** After [^{3}H]proline incorporation into the various groups, collagen was then puri-

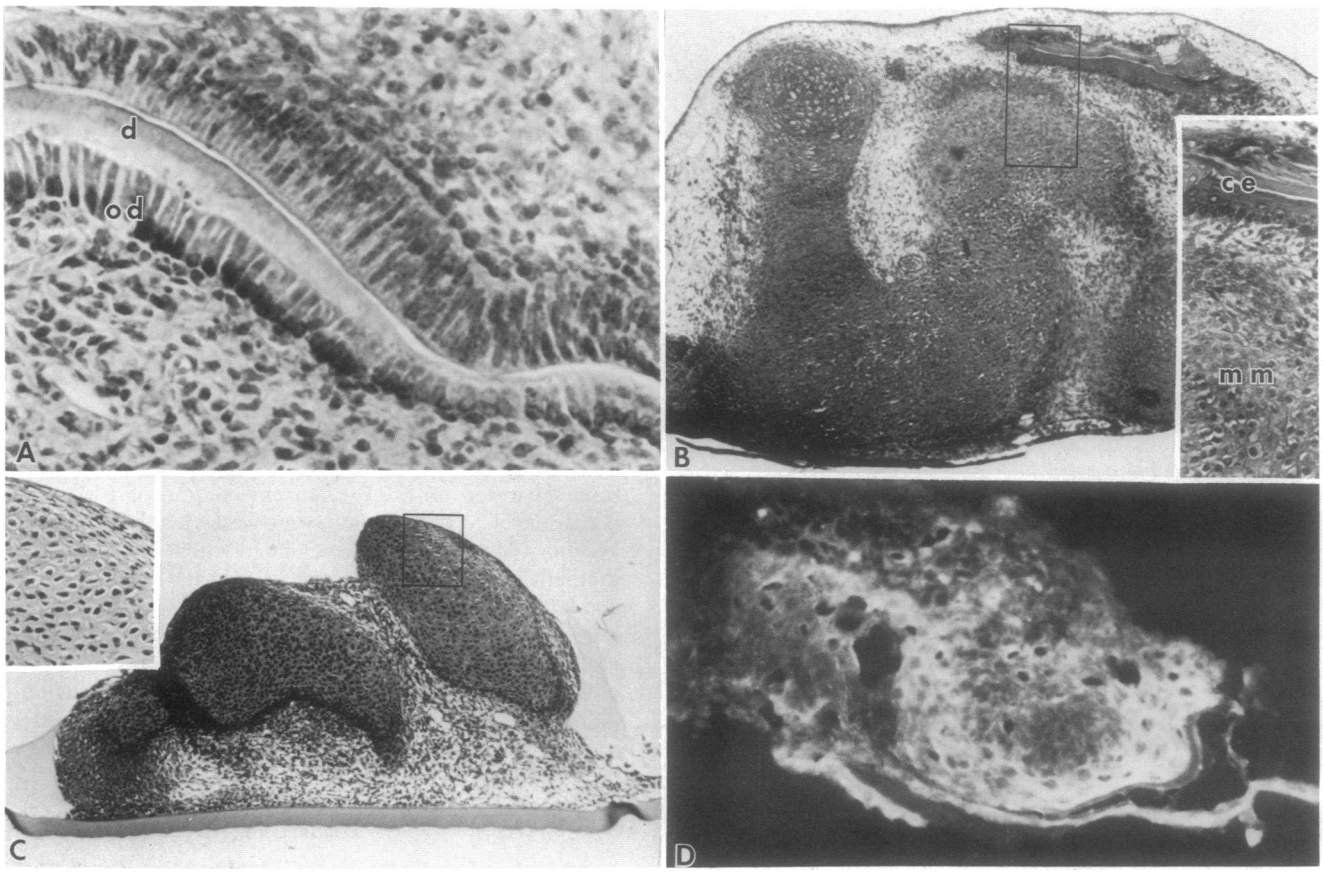

FIG. 2. Heterologous tissue recombinants between chick epithelium and mouse tooth mesenchyme induced cartilage formation in mouse mesenchyme. (*A*) Survey photomicrograph showing histogenesis within tooth explants cultured for 5 days *in vitro*. Mesenchymal cells at the epithelial–mesenchymal interface differentiated into columnar nondividing odontoblasts (od), producing the dentine (d) extracellular matrix. (×650.) (*B*) Chick epithelium (ce) induced mouse mesenchyme (mm) to form cartilage-like structures as recombinants on the chorioallantoic membrane. (×250.) (*Inset*) Interface between heterologous tissues in region indicated by the rectangle. (×700.) (*C*) Structures of cartilage-like cells were also induced *in vitro* within 5 days in recombinants cultured on Millipore filters. (×250.) (*Inset*) Polygonal chondroblasts within the mouse mesenchyme. (×750.) (*D*) Antisera to type II collagen stained the forming cartilage-like regions within the mouse mesenchyme in heterologous tissue recombinants. (×200.)

fied and α-chain compositions were analyzed. Type I collagen is composed of two α_1(I) chains and one α_2 chain, with an α_1:α_2 ratio of 2:1. Type II collagen is composed of three identical chains $[\alpha_1(II)]_3$ differing genetically from α_1(I) chains (22). These ratios were greater than 2:1, indicating synthesis of excess collagen chains in all groups except isolated mouse mesenchyme (Table 1). Furthermore, we observed that intact or recombined epithelial–mesenchymal tissues synthesized more collagen than did isolated mesenchymal tissues alone in culture.

Differential salt precipitation methods separated genetically different collagen molecules, and CM-cellulose-chromatography then separated and purified the genetically distinct collagen subunits. The α_1:α_2 ratio was greater than 2.5 in the 2.6 M NaCl precipitated material (Fig. 3 *A* and *C*), indicating that the majority of synthesized collagen was type I. The supernatant resulting from the differential salt precipitation demonstrated that 70–90% of the collagens were composed of $\alpha_1(I)_3$ type collagen (calculated from the α_1:α_2 ratio) (Fig. 3 *B*

Table 1. Effects of epithelial–mesenchymal interactions on collagen synthesis *in vitro**

Experimental group	Total collagen synthesized, dpm/tissue	DNA content, μg/tissue	Collagen synthesis, dpm/10^3 cells†	α_1:α_2 ratio‡
17-day mouse molar	1,580	2.8	3.6	2.5
Molar tooth organ after 5 days *in vitro*	29,800	2.0	95	3.0
17-day mouse molar mesenchyme	1,540	0.57	17	2.6
Mouse mesenchyme after 5 days *in vitro*	1,620	0.15	69	2.1
Stage 22–23 chick limb epithelium	<25	0.11	—	—
Chick epithelium after 5 days *in vitro*	<25	<0.10	—	—
Mouse mesenchyme + chick epithelium recombinant after 5 days *in vitro*	21,750	0.25	560	2.8

* The data reported were obtained from two different experiments with the exceptions that data for heterologous tissue recombinants is from three experiments.

† Calculated by using DNA content of normal mouse diploid cells (6.4 μg/10^6 cells).

‡ Calculated from CM-cellulose chromatograms. Original data were divided by 2.2 to correct for difference of imino acid contents between α_1 and α_2 chains.

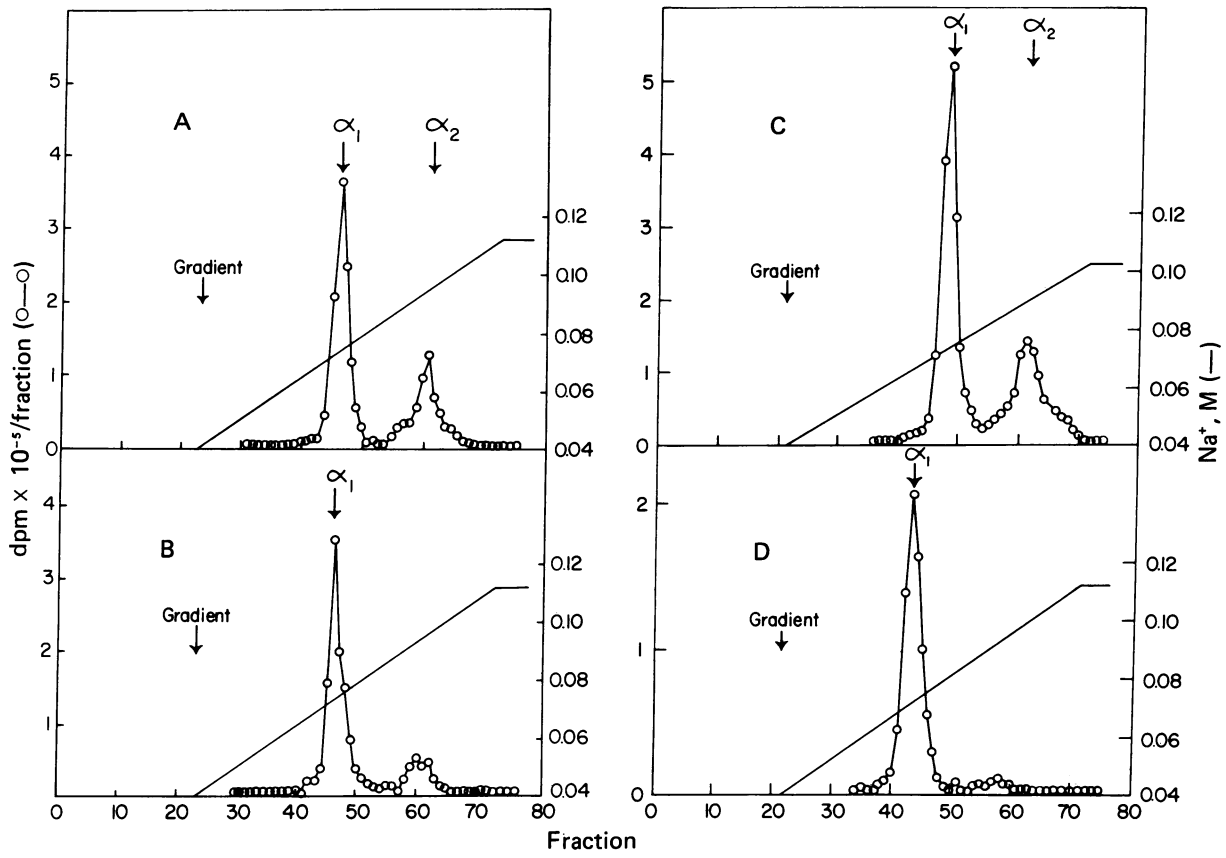

FIG. 3. CM-cellulose chromatography was used to separate collagen α-chains. Radioactive material was purified from molar tooth organs and heterologous tissue recombinants (open circles). Type I and II carrier collagens were used after differential salt precipitation. (*A*) Material synthesized by 17-day embryonic mandibular first molar tooth organs and precipitated at 2.6 M NaCl; (*B*) tooth material that remained in the supernatant; (*C*) material synthesized by heterologous tissue recombinants and precipitated at 2.6 M NaCl; (*D*) heterologous recombinant material that remained in the supernatant.

and *D*). Approximately 30% of the collagen synthesized by tooth organs and by the avian/mouse tissue recombinants in culture was $\alpha_1(I)_3$.

The genetic heterogeneity among synthesized collagens was analyzed by using CNBr cleavage procedures (Fig. 4). The CNBr peptides marked on the chromatograms are based on the nomenclatures of rat α_1 (I) chains (22) and bovine α_1 (II) chains (23, 24). Chromatography of the CNBr peptides from molar tooth or from heterologous recombinant collagens precipitated with 2.6 M NaCl and coincided with α_1 (I) collagen chains (Fig. 4 *A* and *C*). The α_1: α_2 ratios (2.2–2.5) indicated that the major collagen in these fractions was type I $[\alpha_1\ (I)]_2\alpha_2$. The 2.6 M NaCl supernatant from molar tooth collagen coincided with α_1(I) chains (Fig. 4*B*). That fraction contained type I trimer. In contrast, the elution profile of CNBr peptides of synthesized collagens from heterologous recombinants recovered in the 2.6 M NaCl supernatant coincided with type II collagen carrier and small amounts of $\alpha_1(I)_3$.

DISCUSSION

It is demonstrated here that mouse tooth mesenchyme, allegedly a potent inducer of epithelial cytodifferentiation in numerous experimental studies (3, 5, 7, 13, 14, 25–27), differentiated into cartilage in cultures recombined with stage 22–23 avian limb epithelium on the basis of histologic features, immunofluorescence studies, and biochemical identification of type II collagen. As a result of interactions with chicken epithelium, the tooth mesenchymal cells expressed features characteristic of cartilage cells with concomitant increases in collagen synthesis (Table 1).

Several lines of evidence support our conclusion that avian limb epithelium induced *de novo* synthesis of type II collagen in a mesenchymal tissue that normally does not contain type II collagen: (*i*) histological observations showed that chicken epithelium was free of mesenchymal contamination after dissociative procedures and that when cultured alone, this epithelium did not synthesize collagen on the basis of [^{3}H]proline incorporation into collagenase-labile materials; (*ii*) mesenchyme obtained from chicken limb buds and then cultured alone synthesized appreciable amounts of collagen; (*iii*) cartilage cells and their extracellular matrix were observed adjacent and distal to avian epithelium in heterologous tissue recombinations (Fig. 2 *B–C*); (*iv*) neither mouse tooth epithelium nor mesenchyme synthesized type II collagen *in situ* or *in vitro;* (*v*) we identified type II collagen antigens within tooth mesenchyme by using immunohistochemical techniques but were unable to identify type II collagen in either chicken limb or tooth epithelial tissue cultures; and (*vi*) the CNBr cleavage peptides obtained from newly synthesized collagens purified from the 2.6 M NaCl supernatant from heterologous tissue recombinations extracts contained type II collagen (Fig. 4*D*).

These studies demonstrate *de novo* synthesis by a mesenchyme of a unique gene product that was undetectable at the beginning of these experiments. Bernfield (28) had shown that epithelial–mesenchymal interactions during salivary gland morphogenesis enhanced and served to amplify already detectable collagen synthesis *in vitro*, and our studies complement his observations. The present study demonstrates that epithelial–mesenchymal interactions resulted in the *de novo* production of type II synthesis. It is generally assumed that epi-

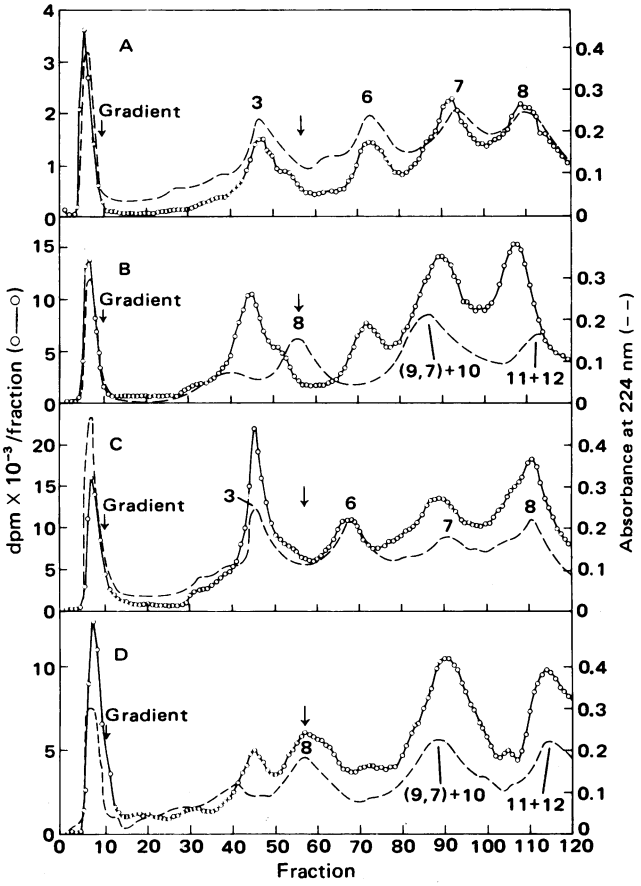

FIG. 4. Analyses of collagen types after CNBr cleavage of differentially salt separated [^{3}H]proline-containing material purified from tooth organs and from heterologous tissue recombinants (open circles). (*A*) Radioactive material synthesized by tooth organs and precipitated with 2.6 M NaCl coincided with CNBr-cleaved type I [α_1(I)] carrier collagen chains (broken line); (*B*) tooth organ material that remained in the supernatant compared with the elution profile of CNBr-cleaved type II [α_1(II)] carrier collagen chains (broken line); (*C*) heterologous recombinants synthesized [^{3}H]proline-labeled material that precipitated with 2.6 M salt is compared with CNBr-cleaved type I [α_1(I)] carrier chains (broken line); (*D*) major peaks of the CNBr-cleaved radioactive supernatant collagen produced from heterologous tissue recombinants coincided with the general profile of the CNBr-digested type II carrier collagen (broken lines).

genetic factors (e.g., mesenchymal factor, nerve growth factor, epidermal growth factor, hormones) function to modulate gene expression in many disparate developing systems (4, 5). We suggest that epithelial–mesenchymal interactions can, under specific conditions, induce *de novo* gene product synthesis as well as regulate rates and relative amounts of synthesis during morphogenesis.

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