Development 105, 697-706 (1989)
Printed in Great Britain © The Company of Biologists Limited 1989

Region-specific deposition of dermal proteins between dermis and epidermis during induction of chick feather and scale rudiments

CHARLOTTE A. PETERSON*, WILLIAM H. PHILLIPS† and ROBERT M. GRAINGER

Department of Biology, University of Virginia, Charlottesville, Virginia, 22901, USA

* Current address: Department of Pharmacology, Stanford University Medical Center, Stanford, CA 94305, USA
† Current address: Abbott Laboratories, Abbott Park, IL 60064, USA

Summary

To begin to study the role of particular proteins in inductive tissue interactions, we have used density labelling techniques to determine whether any dermal proteins are found between embryonic chick dermis and epidermis at a stage when the dermis plays an important inductive role in epidermal differentiation. Epidermis will form feathers or scales depending on whether it interacts with dorsal or foot dermis, respectively, and the dermis can still influence epidermal differentiation when direct cell contact between the tissues is blocked by a membrane filter during culturing (Peterson & Grainger, 1985). In transfilter experiments, we detect a subset of dermal proteins within the filter between the tissues. Several of these dermal proteins are deposited in a region-specific manner, that is, they are only found associated with filters from either dorsal or foot dermis. We have previously shown that the expression of some of these proteins is specific to particular regions of dermis and is also associated with the inductive potential of the dermis (Peterson & Grainger, 1986). We detect only 17 dermal proteins which are transferred across the filter in these cultures and found in direct association with epidermis; of these 14 are common to both dorsal and foot dermis, and 3 are deposited in a region-specific manner. Our results lead us to hypothesize a significant function for certain dermal proteins in this inductive interaction either as part of the extracellular matrix or in direct association with epidermis.

Key words: density labelling, tissue interaction, chick embryo, epidermal differentiation, dermal protein, feather, scale.

Introduction

The dermal–epidermal tissue interaction in the chick embryo results in the morphogenesis of feathers or scales in different regions of epidermis and the synthesis of a specific set of β-keratins characteristic of these two tissues (Dhouailly *et al.* 1978). Although the tissue interactions leading to the formation of feathers and scales are complex, at certain stages the dermis can control the differentiation of the epidermis. By seven days of development, dorsal dermis will induce feather formation and the synthesis of the feather-specific β-keratins in epidermis from any region of the embryo. Similarly, by 13 days, foot dermis will induce scale formation and the synthesis of the scale-specific β-keratins in epidermis from any region (Rawles, 1963). While the molecular basis for this dermal signal is not known, we have shown previously that the different inductive capacities associated with dorsal and foot dermis are reflected in different protein synthetic profiles (Peterson & Grainger, 1986).

One methodology that has been used extensively to help define signalling mechanisms in inductive interactions is the interposition of a membrane filter between interacting tissues. Differentiation occurs in transfilter cultures of dermis and epidermis even when small filter pore diameters (0·2 μm) prevent cell processes from entering the filter and thereby block cell contact between the tissues (Peterson & Grainger, 1985), suggesting that extracellular dermal molecules are involved in this tissue interaction.

Transfilter culturing techniques have been used in attempts to identify specific macromolecules transferred between tissues by radiolabelling one tissue and then following the transfer of labelled molecules across the membrane filter to an unlabelled tissue (Sirlin *et al.* 1956; Pantelouris & Mulherkar, 1957; Grobstein, 1957; Sirlin & Brahma, 1959; Flickinger *et al.* 1959; Grobstein, 1959; Vainio *et al.* 1962; Koch & Grobstein, 1963; Kallman & Grobstein, 1965). Radioactively labelled amino acids have most often been used to prelabel proteins in the inducing tissue. Results of these transfer experiments have not been very useful because they invariably have demonstrated a release by the inducing

tissue of labelled amino acids, which are taken up by the responding tissue and incorporated into protein, making it impossible to determine whether there is protein transfer during these tissue interactions.

Since dermal tissues from different regions of the embryo, with different inductive capabilities, have distinct protein synthetic profiles, we wanted to establish whether any of these tissue-specific proteins are exported from the dermis in transfilter cultures and found either in the filter between the tissues or associated with the epidermis. To avoid the label reutilization problems associated with transfer experiments using radioactive tracers, we have developed a method that involves density labelling. In our experiments, dermis is labelled with dense amino acids and trace amounts of radiolabel, and then placed transfilter to epidermis in unlabelled medium. Dermal proteins within the filter or associated with the epidermis have a heavy buoyant density in an equilibrium salt gradient and can be separated from epidermal proteins. Residual labelled amino acids are still transferred to the epidermis as well; however, these precursors are greatly diluted by unlabelled epidermal amino acid precursor pools. Therefore, proteins synthesized from these pools have a density very close to normal and can be distinguished from dermal proteins which have a heavy buoyant density.

Materials and methods

Culturing and labelling conditions

Fertile White Leghorn chicken eggs (Truslow Farms, Chestertown, MD) were incubated as described previously (Peterson & Grainger, 1985). Pieces of skin 2·5 mm square were obtained from the anterior tarsometatarsus (foot) of 13-day embryos (stage 39, Hamburger & Hamilton, 1951) and from the dorsal feather tract of 7-day embryos (stage 30–31). In some cases, intact skin was labelled with L-[^{35}S]methionine (Amersham, 1400 Ci mmole^{-1}) for 6–8 h as described (Peterson & Grainger, 1986). Dermis and epidermis were separated by mild EDTA treatment (Peterson & Grainger, 1985) and protein prepared from each tissue for equilibrium gradient analysis. Dermis labelled in this way was also used in transfilter culturing experiments similar to those described below.

For density labelling experiments, intact skin from the stages described above was prelabelled in medium composed of the following: 1 mg ml^{-1} ^{13}C^{15}N^{2}H-labelled amino acids (Merck, Sharp and Dohme, Canada Limited, Montreal; isotopic substitution of 80% ^{13}C for ^{12}C, 90% ^{15}N for ^{14}N, and 98% ^{2}H for H) and 300 μCi ml^{-1} L-[^{35}S]methionine (Amersham, 1400 Ci mmole^{-1}) or 125 μCi ml^{-1} L-[U-^{14}C]leucine (Amersham, 300 mCi mmole^{-1}). Due to the loss of acid-labile amino acids during preparation of density-labelled amino acids, the medium was supplemented with 0·1 mM-cysteine, 0·05 mM-trytophan, and 2·0 mM-glutamine. The medium also contained Earles balanced salt solution supplemented with 1 mg ml^{-1} [^{13}C]glucose (grant from the Division of Research Resources, Los Alamos National Laboratory), Basal Medium Eagle (BME) vitamins (Gibco), antibiotic-antimycotic (Gibco), 2·5 mg ml^{-1} $NaHCO_3$, 5% heat-inactivated chicken serum (Gibco) and 5% embryo extract (Peterson & Grainger, 1985). [^{13}C]glucose, although included in all of our experiments, does not substantially increase the density shift of proteins in these experiments. Serum and embryo extract were dialysed for 8 h against Earles balanced salt solution at 4 °C to deplete them of low molecular weight molecules, such as amino acids, that might reduce the incorporation of stable isotopes. Skin was density labelled for 24 h at 37 °C in a 5% CO_2–95% air atmosphere in a humidified incubator. Dermis was then separated from the epidermis and washed in standard culture medium composed of BME supplemented with Modified Eagle Medium essential and non-essential amino acids (Gibco), 5% (v/v) inactivated chicken serum (Gibco), and 5% (v/v) embryo extract. After washing, foot dermis was combined transfilter with unlabelled foot epidermis and dorsal dermis was combined transfilter with unlabelled dorsal epidermis as previously described (Peterson & Grainger, 1985), and cultured in standard culture medium. In some experiments, 100 μCi ml^{-1} L-[4,5-^{3}H]leucine (Amersham, 52 Ci mmole^{-1}) was added. In all transfilter experiments, 0·2 μm pore diameter Nuclepore filters were used. Tissues did not adhere to or penetrate the filters as judged by transmission and scanning electron microscopy (Peterson & Grainger, 1985) and were easily lifted off for the preparation of proteins for equilibrium gradient analysis as described below.

Equilibrium density centrifugation of protein

After [^{35}S]methionine or density labelling, each epidermis or dermis was homogenized in 80 μl of a 1·15 g ml^{-1} solution of KSCN (Sigma) in H_2O for gradient analysis. In transfilter experiments where protein was recovered from the gradients, epidermis from 6 cultures was pooled and homogenized in the same way for gradient analysis. Similarly, filters from 6 transfilter cultures were pooled and added to the KSCN solution to solubilize proteins trapped within the filters. Approximately 70% of the total labelled protein, determined by recovery of trichloroacetic acid (TCA) precipitable cts min^{-1}, was soluble in the KSCN solution and no preferential protein loss was observed (data not shown). Each 80 μl sample was added to the following mixture of salts: 883 μl of a 1·43 g ml^{-1} solution of KSCN in H_2O, 212 μl of a 1·52 g ml^{-1} solution of CsSCN (Syn-Met, Inc., Baton Rouge, LA) in H_2O, 261 μl H_2O, and 60 μl of a 10 mg ml^{-1} solution of both lysine and glutamine in 1·0 M-Tris–HCl (pH 7·0). These 1·5 ml gradients have a density of 1·32 g ml^{-1}. Gradients were centrifuged to equilibrium in Beckman polyallomer tubes overlaid with light paraffin oil in a Beckman SW 60 Ti rotor at 58 000 rev min^{-1}, for 60 h at 5 °C.

After centrifugation, three drop fractions were collected yielding a total of 27–30 fractions. At equilibrium, a shallow, linear gradient is generated in which the density ranges from 1·44 g ml^{-1} at the bottom of the tube to 1·26 g ml^{-1} at the top. From each fraction, 3 μl samples were counted in a LKB Racbeta 1215 scintillation counter and selected fractions were pooled for recovery of protein by TCA precipitation, followed by polyacrylamide gel electrophoresis (PAGE) analysis.

Electrophoresis of proteins

One-dimensional SDS–polyacrylamide (1-D SDS–PAGE) gels were run essentially according to the methods of King & Laemmli (1971) except that the separating gel was a 5–22·5% gradient of acrylamide. Molecular weight standards were obtained from Biorad Laboratories. Gels were prepared for fluorography as described by Bonner & Laskey (1974) and were exposed to Kodak XAR film for one week. Two-dimensional (2-D) gels were run essentially according to the methods of Garrels (1979) as described elsewhere (Peterson & Grainger, 1986), except that protein samples recovered from

gradients were resuspended directly in Garrels' sample buffer containing 3 % SDS. After transfilter culturing and gradient analysis, dense protein from 36 epidermal tissues or filters was pooled and run on a single gel. Unlabelled protein markers (Electrophoresis Calibration Kit, Pharmacia) were also loaded on each gel to aid in alignment of gels during analysis of protein spots. After electrophoresis, gels were stained with 0·1 % (w/v) Coomassie Brilliant Blue R and the migration of the protein markers was compared. Gels were then prepared for fluorography as described by Bonner & Laskey (1974), and exposed to film for 6 months. All experiments were performed at least twice.

Results

Equilibrium gradient analysis of dermal and epidermal proteins

Using previously described gradient techniques (Boudet *et al.* 1975; Leffak *et al.* 1977; Fourcroy, 1978; Salhanick *et al.* 1983), it was not possible to obtain the narrow, well-resolved peaks of dense and light total cellular protein during equilibrium centrifugation required for our experiments. In order to achieve such resolution, we have developed a gradient system utilizing KSCN and CsSCN salts (Phillips *et al.* 1989). Proteins are denatured and extremely soluble in these salts, and shallow, linear gradients are produced in which normal and density-labelled whole-cell protein extracts form sharp, well-resolved peaks. Protein from most tissues we have tested forms a single peak at the normal buoyant density in this gradient system, as does the protein from 7-day dorsal chick epidermis (Fig. 1A).

When seven-day dorsal dermal protein is centrifuged in KSCN/CsSCN gradients, however, it is resolved into two distinct peaks (Fig. 1B), one with the expected buoyant density and one with a higher buoyant density. Similar results are obtained with 13-day foot tissues (data not shown). Recovery of protein from the two dermal gradient peaks and analysis by 1-D SDS–PAGE indicates that the lighter peak contains most of the tissue proteins (Fig. 1C, lane 2) and the dense peak is composed predominantly of two proteins which migrate with apparent relative molecular masses of near 100×10^3 (Fig. 1C, lane 1). Higher molecular weight multimers are also present. These dense proteins are not detected among proteins synthesized in the epidermis (Fig. 1C, lane 3). Similar protein profiles were obtained with foot dermis and epidermis (data not shown).

The abundant proteins in the dense dermal peak of Fig. 1B are likely to be collagen. Due to their abundance in the dermis they are probably subunits of type I collagen. Type I collagen is composed of two α1(I) chains and one α2(I) chain, each with reported relative molecular masses of approximately 95×10^3. When electrophoresed under denaturing conditions, the α1(I) chains migrate with an apparently higher molecular weight (Miller, 1984). It is also known that if crosslinking is not completely blocked during extraction, higher molecular weight dimers, trimers, and larger aggregates of the collagen chains are produced. The proteins in the dense region of the gradient have these properties. Studies on proline incorporation and collagenase digestion (data not shown), also support our tentative

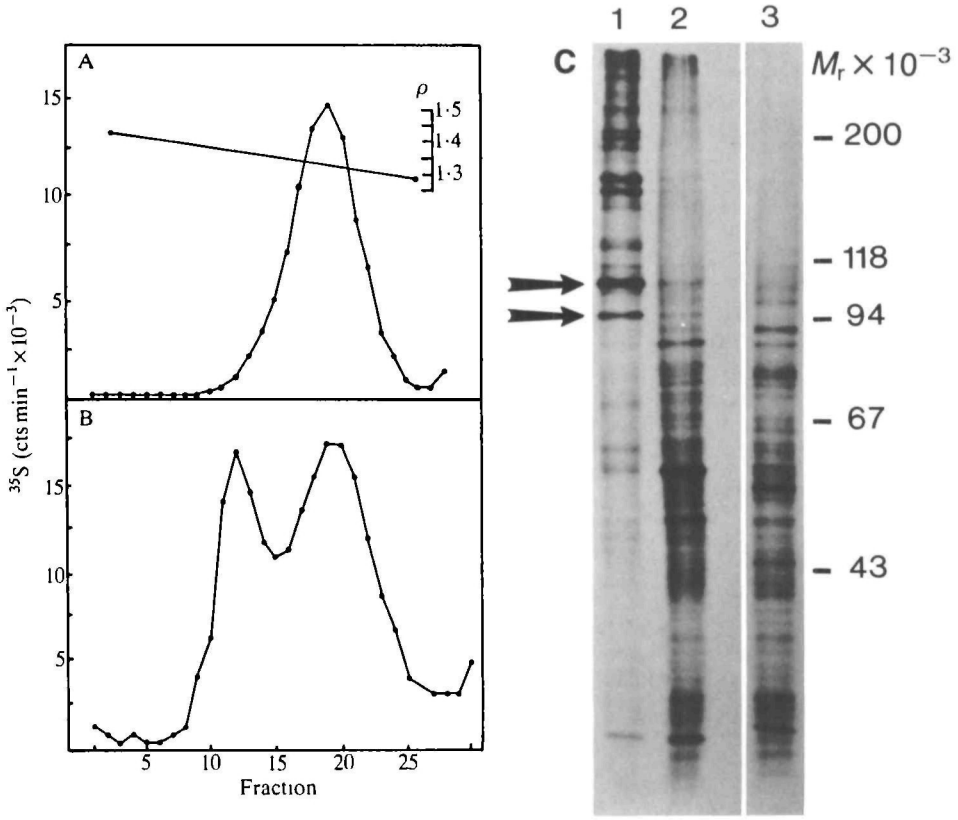


Fig. 1. Analysis of [^{35}S]methionine-labelled 7-day dorsal dermal and epidermal protein. (A) KSCN/CsSCN gradient profile of epidermal protein. (B) KSCN/CsSCN gradient profile of dermal protein. The density distribution across this gradient is identical to the one shown in (A). (C) One-dimensional SDS gel of proteins present in gradient peaks from A and B with relative molecular mass markers. Lane 1: protein profile from the dense dermal peak in B, fractions 7–14. Arrows indicate the 2 prominent bands which migrate with apparent relative molecular masses of approximately 100×10^3. Lane 2: protein profile from the light dermal peak in B, fractions 15–25. Lane 3: protein profile from the epidermal peak in A, fractions 9–26. Approximately 50 000 cts min^{-1} of protein were loaded per well and gels were exposed to film for 1 week.

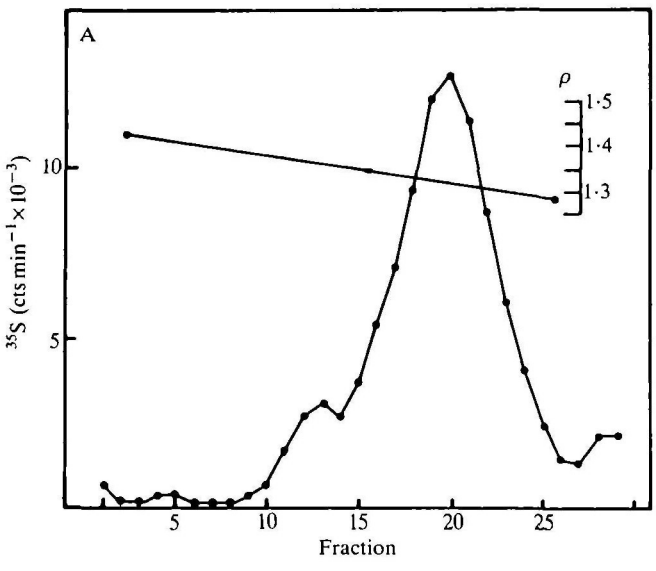
identification of these dense dermal proteins as collagen.

Are particular dermal proteins found between dermis and epidermis in transfilter cultures?

Although density labelling was used to follow export of most dermal proteins, the inherently high buoyant density of the dermal proteins tentatively identified as collagen allowed us to perform a density transfer experiment, without density label, to determine whether these proteins are found in association with epidermis. In fact this was the only way to follow these proteins since in the heavy isotope experiments described later these proteins were so dense that they pelleted in our gradients making it impossible to follow them during the course of such an experiment. To monitor transfer of the intrinsically dense dermal proteins, we performed a transfilter experiment similar to the density-labelling experiments described below, except that the dermis was labelled only with [^{35}S]methionine. Appearance of a dense peak of protein associated with the epidermis after transfilter culturing with an [^{35}S]methionine-labelled dermis would argue that some of the intrinsically dense protein fraction was transferred from the dermis. Results of such an experiment using dorsal tissues are shown in Fig. 2. Equilibrium centrifugation of epidermal protein after 24 h of culture transfilter from an [^{35}S]methionine-labelled dermis demonstrates that the epidermis contains both light and dense protein components (Fig. 2A). The protein present in the dense peak was analysed by 1-D SDS–PAGE (Fig. 2B, lane 2). This peak contains proteins which comigrate with the dense proteins synthesized by the dermis (see Fig. 1C, lane 1). These proteins must have been synthesized by the dermis and transferred to the epidermis since epidermis labelled directly with [^{35}S]methionine (Fig. 2B, lane 1) or cultured transfilter from dermis (see Fig. 4, below) does not synthesize detectable levels of these dense proteins.

While this experiment was useful for following the intrinsically dense dermal component, one clearly cannot determine the origin of the proteins in the light peak associated with the epidermis. They may have been synthesized by the epidermis utilizing precursor [^{35}S]methionine which was transferred across the filter from the dermis or by transfer of labelled protein from the dermis which has the same buoyant density as epidermal protein.

Density labelling experiments were required to determine whether any of the dermal proteins that normally have a light buoyant density might be found between dermis and epidermis in transfilter experiments. Because we expected that many proteins exported from the dermis might be found in the extracellular matrix (ECM) between the two tissues, we analysed dense proteins within the filter, as well as those associated directly with the epidermis. In these experiments, 7-day dorsal or 13-day foot dermis was labelled with $^{13}C^{15}N^{2}H$-substituted amino acids and trace amounts of [^{35}S]methionine (to follow incorporation of density label) for 24 h. Dermal protein was extensively density labelled as determined by equilibrium centrifugation (Fig. 3A). After the initial labelling, dermis was combined transfilter with unlabelled epidermis and cultured 24 h in unlabelled medium. Feather-like or scale-like morphogenesis in overlying epidermis began within 24 h of transfilter culturing with a labelled dermis (data not shown); however, epidermal appendages generally did not differentiate to the same extent as those in control cultures (Peterson & Grainger, 1985). After transfilter culturing, gradient analysis of protein present in both the filter between the tissues (Fig. 3B) and the epidermis (Fig. 3C) revealed two peaks of protein. Far more dense protein is found within the filter than associated with the epidermis itself. The appearance of a dense peak in both gradients suggests that some density-labelled proteins may have been transferred from the dermis to the epidermis. The protein in the peaks of



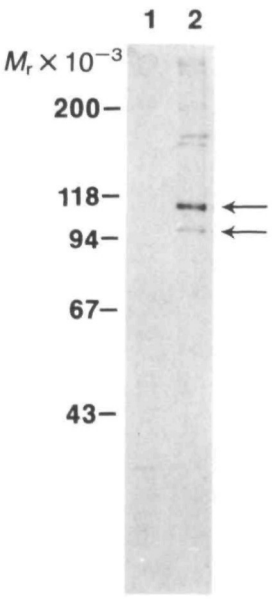


Fig. 2. Analysis of protein transfer from 7-day dorsal dermis to epidermis without density labelling. (A) KSCN/CsSCN gradient profile of protein present in the epidermis after transfilter culturing. (B) One-dimensional gel analysis of protein recovered from equilibrium gradients with relative molecular mass markers. Lane 1: proteins present in the dense region of a gradient from an epidermis labelled directly with [^{35}S]methionine (for example, Fig. 1A, fractions 7–14). Lane 2: proteins present in the dense region of a gradient from epidermis after transfilter culturing with [^{35}S]methionine-labelled dermis (fractions 8–14, from A). Arrows indicate transferred proteins tentatively identified as collagen.

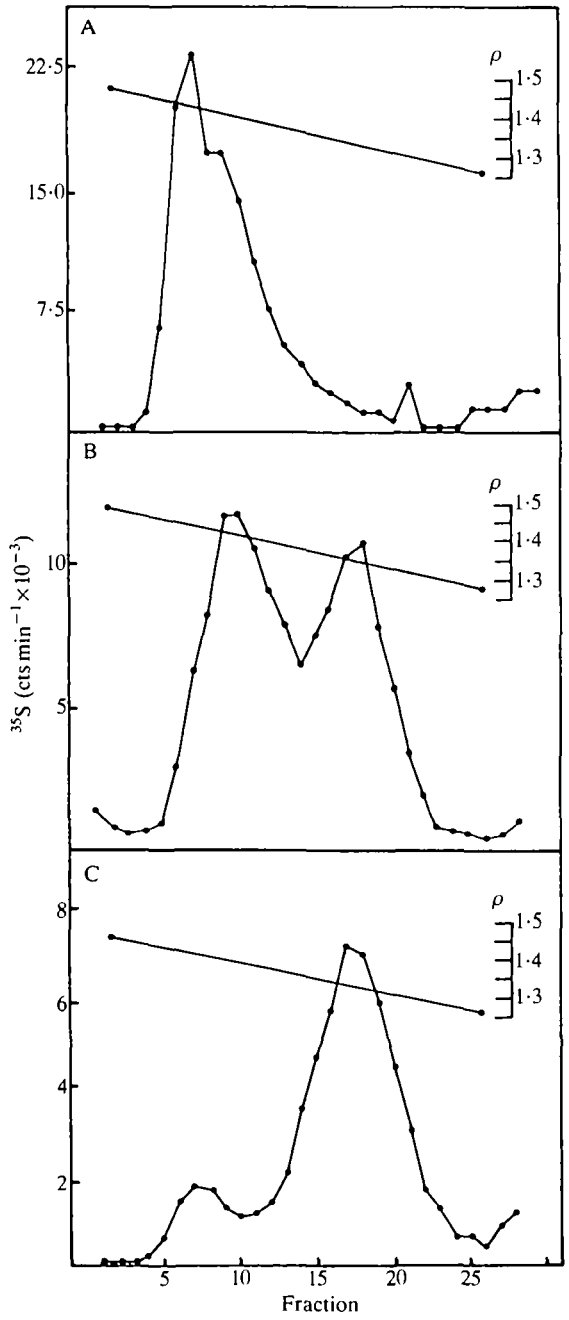


Fig. 3. Gradient analysis of dorsal dermal and epidermal protein from a density-labelling experiment. (A) KSCN/CsSCN gradient profile of density labelled dermal protein prior to transfilter culturing. (B) KSCN/CsSCN gradient profile of protein present in the filter between the tissues after 24 h of transfilter culturing. (C) KSCN/CsSCN gradient profile of protein present in association with the epidermis after 24 h of transfilter culturing. Proteins present in the dense regions of the gradients in B and C were analysed on 2-D gels (Fig. 5).

normal buoyant density in Fig. 3B and 3C may have been synthesized in the epidermis utilizing residual precursor [^{35}S]methionine transferred across the filter, or may have originated from dermal protein synthesized during transfilter culturing in light medium.

Although the peak of protein present in the dense region of both gradients is consistent with its origin from the dermis, it may have been synthesized in the epidermis by reincorporation of residual density-labelled precursors. Several lines of evidence strongly argue that the dense protein peaks seen in Fig. 3B and C are of dermal origin. First, since dense amino acid precursor pools are being diluted during transfilter culturing in light medium, reincorporation of these precursors in the epidermis to produce entirely dense protein might be expected to occur only at relatively early times during the culture period. However, gradient analysis of the epidermis at earlier time points, that is, 12 and 18 h after the onset of transfilter culturing, indicates no detectable dense protein peak (data not shown). This pattern (no dense protein at 12 or 18 h, followed by a small amount of dense protein at 24 h) could conceivably be due to slow incorporation of density label by the epidermis during a very poor chase. Double radiolabel experiments were performed (Fig. 4) which show that this is not the case: the chase is both rapid and effective. Seven-day dorsal or 13-day foot dermis was labelled with $^{13}C^{15}N^{2}H$-substituted amino acids and [^{14}C]leucine (to follow incorporation of density label). After 24 h, this dermis was placed transfilter from an unlabelled epidermis and cultured in standard culture medium supplemented with [^{3}H]leucine. Equilibrium gradient analysis of epidermal protein after 2 h (Fig. 4), 6 h, and 12 h of transfilter culturing resulted in similar gradient profiles. All of the newly synthesized ^{3}H-labelled, epidermal protein has a normal buoyant density. ^{14}C- or density-labelled precursors transferred to the epidermis are also incorporated into protein of normal buoyant density demon-

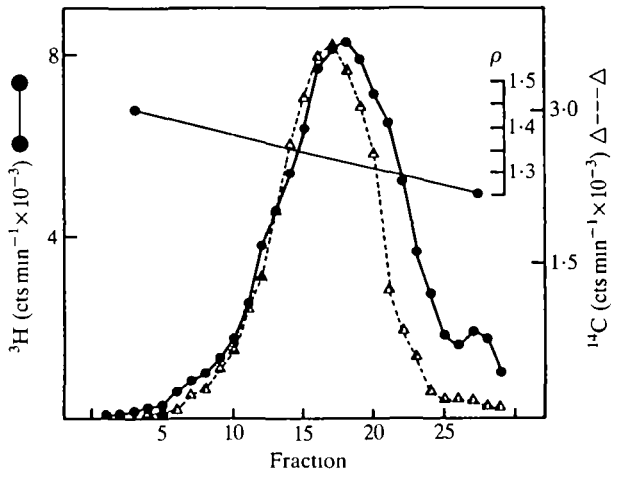


Fig. 4. Gradient analysis of protein synthesized in the epidermis during culturing transfilter from a density-labelled dermis. Seven-day dorsal dermis was labelled with $^{13}C^{15}N^{2}H$-substituted amino acids and [^{14}C]leucine for 24 h. This dermis was then combined transfilter with unlabelled 7-day epidermis and cultured in standard culture medium supplemented with [^{3}H]leucine for 2 h. Protein from each tissue was then analysed by equilibrium centrifugation. ●——●, gradient profile of ^{3}H-labelled protein; △---△, gradient profile of ^{14}C-labelled protein.

strating that they are rapidly and extensively diluted by chase medium and endogenous epidermal amino acid precursor pools. Since dense proteins are detected in association with the epidermis only after 24 h of transfilter culture we conclude that they must have originated from the dermis.

Analysis of dense protein recovered from gradients following density labelling and transfilter culturing

Proteins recovered from the dense regions of the gradients illustrated in Fig. 3 were analysed by 2-D SDS–PAGE (Fig. 5). Fig. 5A and B are fluorograms of dense proteins present in the filter between 7-day dorsal dermis and epidermis, and 13-day foot dermis and epidermis, respectively, after a density-labelling experiment. Only a small subset of dermal proteins is detectable in the filters between the tissues. Most proteins are common to both these filters, but two are reproducibly present only in the dorsal filter (1 and 5). Protein 1 comigrates with a protein normally synthesized only in dorsal dermis (Fig. 6A), and not in foot dermis (Fig. 6B). Protein 5 is synthesized by both dorsal and foot dermis, but is only found associated with filters of dorsal transfilter cultures (Fig. 5A). Two proteins present in the filter between dermis and epidermis (2 and 3) comigrate with proteins previously identified as being specific to foot dermis (Peterson & Grainger, 1986). These proteins are not detectable in

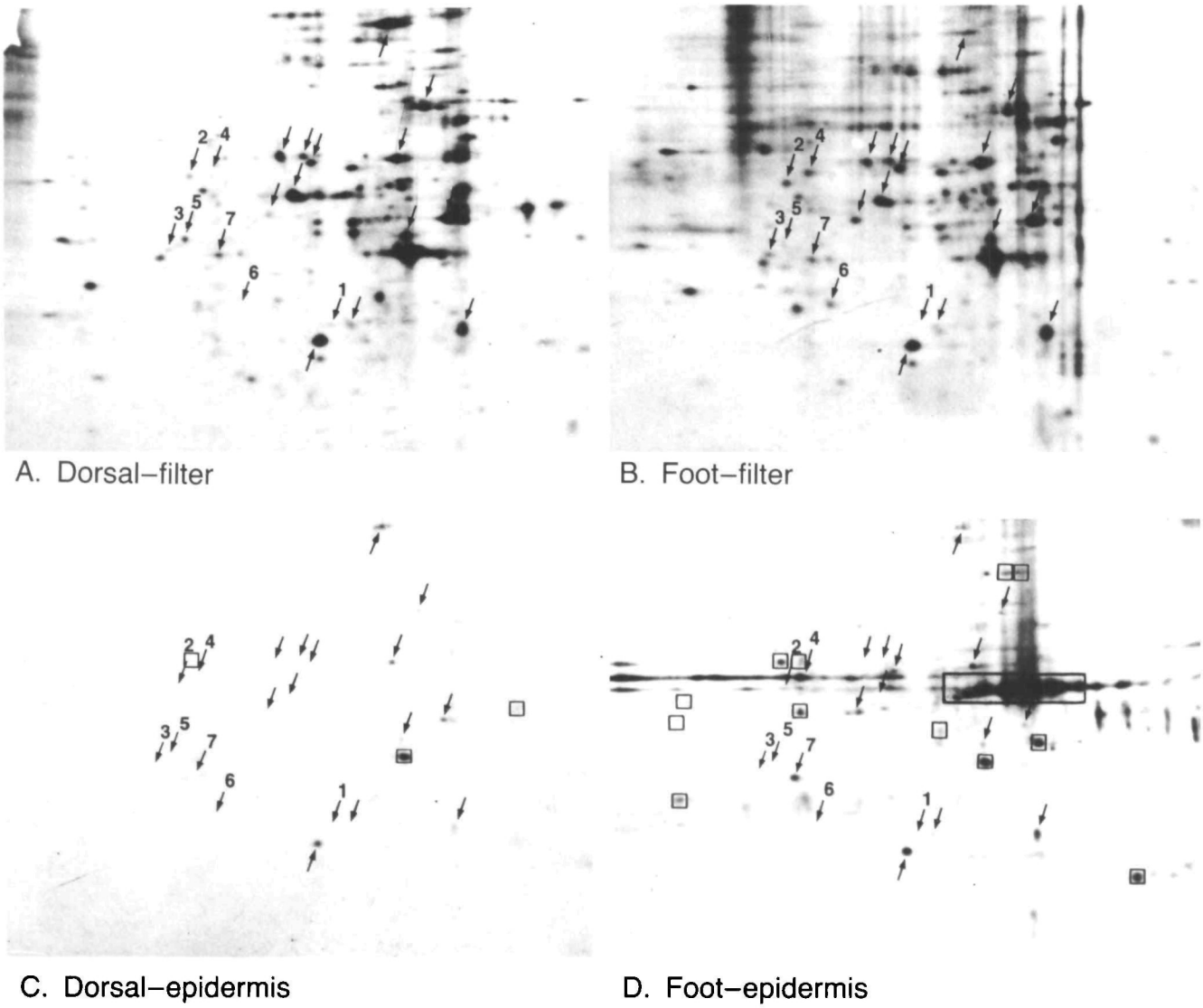


Fig. 5. Two-dimensional gel analysis of [^{35}S]methionine-labelled proteins recovered from the dense region of equilibrium gradients after a density-labelling experiment. Dense protein found in the filter between dorsal (A) and foot (B) dermis and epidermis. Dense protein found associated with dorsal (C) and foot (D) epidermis. Unnumbered arrows indicate dense proteins reproducibly associated with the epidermis. Protein 1 is specific to dorsal dermis and is detectable in the filter between the tissues. Proteins 2 and 3 are enriched in foot dermis and are detectable in the filter between both dorsal and foot tissues. Proteins 4 and 6 are common to dorsal and foot dermis but are exported only by foot dermis. Protein 5 is common to dorsal and foot dermis but is exported only by dorsal dermis. Protein 7 is present in the filter between both dorsal and foot tissues, but is only detectable associated with foot epidermis. This protein does not comigrate with any identifiable dermal protein. Boxes delineate epidermally synthesized proteins (see Fig. 7).

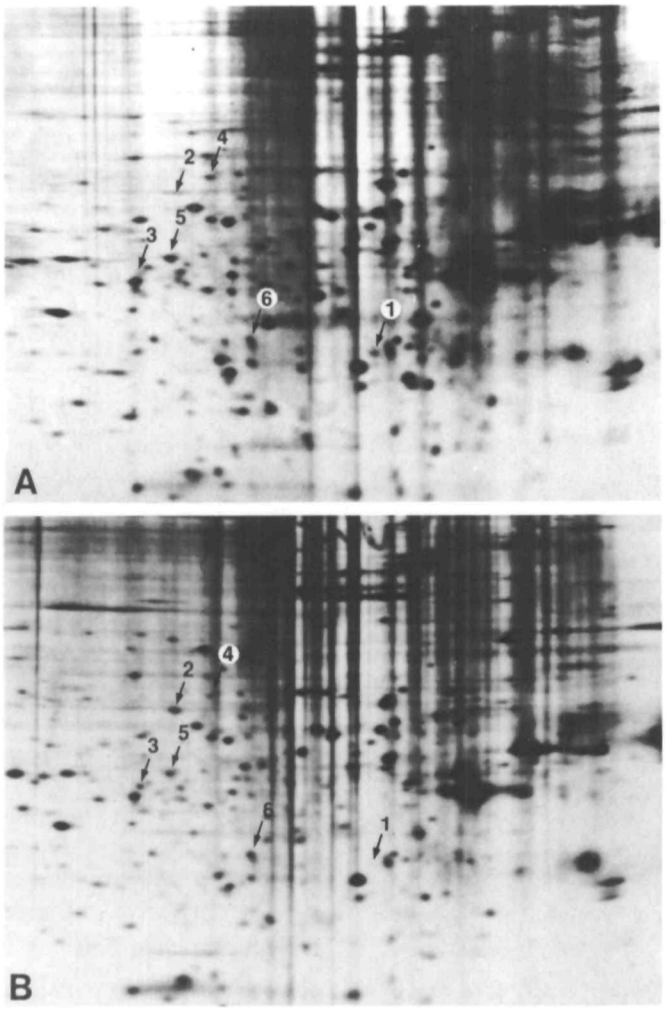


Fig. 6. Two-dimensional gel analysis of total [^{35}S]methionine-labelled dermal protein. (A) Dorsal dermal protein. (B) Foot dermal protein. Numbers indicate proteins that comigrate with dense proteins found in the filter and associated with the epidermis after a density-labelling experiment (see Fig. 5).

dorsal dermis (Fig. 6A); however, they are clearly present in the filter between dorsal tissues, although less abundant than between foot tissues (compare Fig. 5A and B). It would appear that both proteins are synthesized at low levels by the dorsal dermis and highly concentrated in the extracellular space between the dermis and epidermis. Proteins 4 and 6 are normally synthesized by both dorsal and foot dermis (Fig. 6A and B), but are only detected in the filter between foot dermis and epidermis (compare Fig. 5A and B). One protein common to both filters (7) does not appear to comigrate with any identifiable dermal protein.

Only a fraction of the dense protein detectable in the filters is also reproducibly found to be associated with dorsal (Fig. 5C) or foot (Fig. 5D) epidermis. The remainder of the proteins may be at concentrations below our level of detection in the epidermis or may be incorporated into the ECM and not associated tightly with the epidermis. Among the dense proteins that we do detect, 13 proteins are associated with both dorsal and foot tissues (indicated in Fig. 5A–D by unnumbered arrows). Neither proteins 1 and 5, associated with the dorsal filter, nor protein 6 associated with the foot filter, are detectable in association with the epidermis (Fig. 5C and D). However, protein 4, which is found only in the filter between foot dermis and epidermis, appears to be associated with foot epidermis, though it is somewhat obscured by a protein smear in this region (Fig. 5D). Proteins 2 and 3, found in the filters between both dorsal and foot tissues, are not detectable in association with dorsal epidermis (Fig. 5C). Protein 2 does appear to be present in association with foot epidermis, though it is also partially obscured by a protein smear in this region (Fig. 5D). While protein 7 is present between the tissues from both regions (Fig. 5A and B), it is found to be associated only with foot epidermis (Fig. 5D). Although equal amounts of material were loaded on the gels for Fig. 5C and 5D, the amount of radioactivity in Fig. 5C appears somewhat reduced. Therefore, we cannot exclude the possibility that proteins, such as 7, which appear to be foot-specific, are also found at low levels in association with dorsal epidermis. As mentioned earlier, proteins with inherently high buoyant densities present in the dermis (see Figs 1 and 2) pelleted in the gradient after density labelling, so we did not detect the transfer of these proteins from dermis to epidermis in these experiments.

Since there is a slight overlap between dense and light protein peaks, and because a few proteins have aberrant buoyant density properties, some epidermal proteins may have contaminated the dense protein peak in these experiments. We wanted to determine if any proteins visualized on the 2-D gels of dense proteins (Fig. 5A–D) were actually epidermal proteins which had banded in the dense protein region. To test this possibility, [^{35}S]methionine-labelled 7-day dorsal and 13-day foot epidermal proteins were centrifuged in KSCN/CsSCN gradients and proteins were isolated from the gradient fractions corresponding to the dense protein region (for example, Fig. 1A, fractions 7–14) and then analysed by 2-D PAGE (Fig. 7A and B). There are relatively few epidermal proteins in the dense region and none appear to comigrate with the proteins identified as being of dermal origin. These epidermal proteins are delineated by boxes in Fig. 7 and in Fig. 5.

Discussion

The combination of transfilter culturing and density-labelling techniques has enabled us to determine whether dermal proteins are found between dermis and epidermis during an important inductive interaction between these tissues. Grainger & Wessells (1974) used similar methods to ask if RNAs are transferred between tissues during a developmental tissue interaction. Using density-labelled nucleosides to prelabel the inducing tissue, mouse lung mesenchyme, no transfer of RNA to the responding epithelium was detected. Toxic effects of heavy isotopes on developing tissues were minimal,

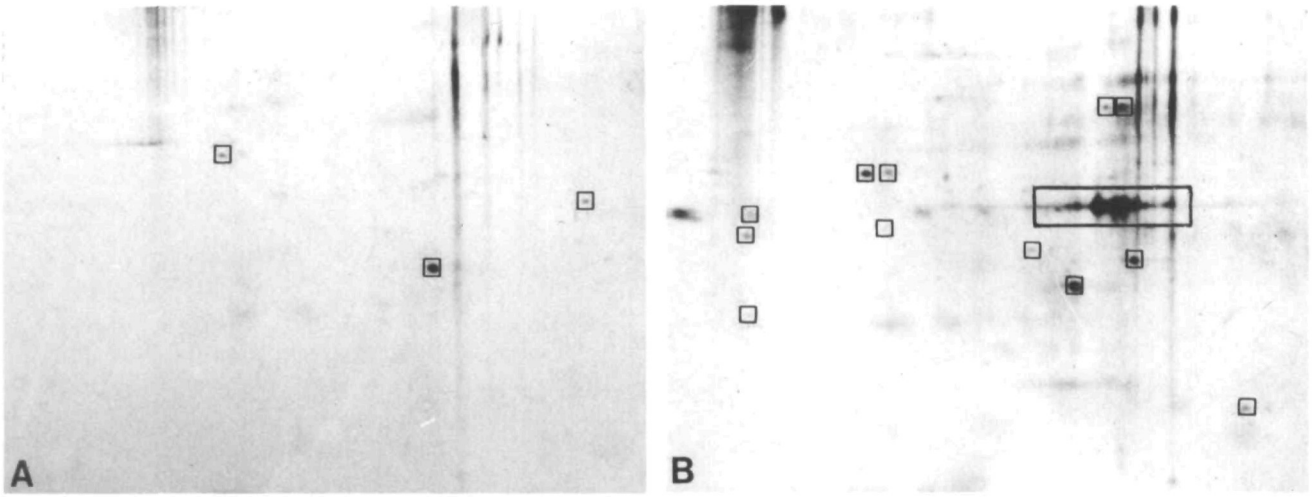


Fig. 7. Two-dimensional gel analysis of [^{35}S]methionine-labelled epidermal proteins banded in a CsSCN/KSCN gradient and isolated from the dense side of the gradient peak. [^{35}S]methionine-labelled dorsal (A) and foot (B) epidermal protein collected from the dense region of a gradient (for example, Fig. 1A, fractions 7–14). Proteins reproducibly recovered from this region of the gradient are delineated by boxes.

and morphogenesis proceeded in a manner closely resembling *in vitro* morphogenesis in control cultures. We find that chick dermis prelabelled in the presence of dense amino acids subsequently induces feather-like or scale-like differentiation in overlying epidermis, and we do find dermal proteins between the dermis and epidermis during this interaction. However, we are probably not assaying all dermal proteins that are in this class. We can only monitor those proteins synthesized during the density-labelling period; any proteins synthesized earlier during development and exported from the dermis would not be detected. In addition, we can detect only those proteins that are stable during the experiment. We are also visualizing only those relatively abundant proteins that are resolved by our gradient and gel systems.

In the present study, we demonstrate the transfer to the epidermis of 14 proteins common to both dorsal and foot dermis, the most abundant of which is likely to be collagen. The fact that only a subset of dermal proteins appears to be transferred confirms our finding that contact between the tissues does not occur in transfilter cultures (Peterson & Grainger, 1985).

We have also identified region-specific proteins exported from the dermis, primarily localized within the filter between the tissues. These proteins fall into several categories.

(A) Protein 1, which is exported from dorsal dermis, appears to be specific to this region of the dermis (Peterson & Grainger, 1986). This protein is also missing from apteric dermis, which is unable to induce epidermal appendage formation. In addition, protein 1 is synthesized by *young* foot dermis, which induces feather formation when combined with dorsal epidermis. Synthesis of this protein ceases at the time foot dermis acquires the ability to induce scales in dorsal epidermis (Peterson & Grainger, 1986), implying further that it may play a functional role in this tissue interaction.

(B) Two proteins enriched in foot dermis (2 and 3) are found at higher levels between foot tissues than dorsal tissues. Whether such quantitative differences in regional localization of dermal proteins may have significant effects on the interacting tissues is not clear. Protein 2 also appears to be associated with foot epidermis.

(C) Three proteins are synthesized by both dorsal and foot dermis, but are found only in filters between foot tissues (4 and 6), or dorsal tissues (5). Protein 4 also appears to be associated with foot epidermis. This localization would appear to be due to some kind of secondary process, perhaps a region-specific dermal or epidermal factor affecting protein stability.

(D) One protein (7) is present in the filters between the tissues from both regions, but is only found to be associated with foot epidermis. Region-specific protein stability may also be involved in this case. This protein does not comigrate with any identifiable dermal protein. Since these experiments enrich for the small subset of dermal proteins transferred between tissues, it allows detection of proteins not normally discerned on 2-D gels of total dermal protein (for example, proteins 2 and 3 are not seen in gels of total dorsal dermal proteins, but are detectable in samples from the filter between dorsal dermis and epidermis). Therefore, protein 7 may be synthesized in the dermis but be too rare to detect on 2-D gels of total dermal protein. Alternatively, this protein may be structurally modified in the ECM causing it to migrate differently on 2-D gels. While there is no simple region-specific pattern to the dermal proteins found between dermis and epidermis, the pattern is regulated and suggests that an underlying mechanism involving some of these proteins in inductive effects is plausible.

Most of the proteins found in the filter between the dermis and epidermis are not subsequently detectable in association with the epidermis. The incomplete differentiation of the epidermis in transfilter cultures

may be partially due to the fact that important proteins do not traverse the filter efficiently. Alternatively, if the function of these proteins was to provide a unique organization to the ECM between dermis and epidermis, we might not expect them to be associated with the epidermis during these experiments.

The organization of the ECM is clearly important for skin differentiation. Stuart & Moscona (1967), Goetinck & Sekellick (1972), and Stuart *et al.* (1972) have proposed that the organization of the collagen lattice within the dermis establishes the site of dermal condensation formation and therefore feather outgrowth. It has also been proposed that in dorsal chick skin, collagen has a stabilizing effect on epidermal differentiation since as feather rudiments begin to form, the amount of collagen within the dermal feather papilla decreases, while it increases in surrounding interplumar dermis (Kitamura, 1981; Mauger *et al.* 1982; Demarchez *et al.* 1984). This differential organization of collagen, which may be functionally significant in the dermal control of epidermal appendage patterning and morphogenesis, may be produced through interactions with other protein components of the ECM. The regionally specific proteins we have identified may provide variation to the organization of the collagen lattice between the tissues, and the epidermis may respond by forming feather or scale rudiments in the appropriate pattern.

Work on tooth (Kollar, 1983) and bone (Reddi, 1983) development also supports the idea that ECM proteins associated with collagen may provide specificity to the organization of the matrix. Kollar has demonstrated that collagen production by dental mesenchyme is required for tooth induction in the epithelium. He proposes that the ECM is organized into specific patterns depending on the variety and proportion of molecules, in addition to collagen, that are present, and this matrix pattern is important for tooth differentiation in the epithelium. Reddi (1981) has reported that subcutaneous implantation of extracellular bone matrix results in the development of endochondral bone and cartilage by surrounding tissues. The implanted ECM is predominantly collagen and some tightly associated glycoproteins. These glycoproteins, with relative molecular masses of less than 50×10^3, when reconstituted with inactive collagenous matrix, will induce bone formation *in vivo* (Sampath & Reddi, 1981) and cartilage formation from muscle *in vitro* (Sampath *et al.* 1984). Reddi proposes (1983) that these glycoproteins may have instructive capabilities to complement the less-specific inductive effects of the majority of the ECM molecules.

Our density-labelling experiments have been essential for demonstrating that a specific subset of dermal proteins is found between dermis and epidermis during feather and scale formation, and argue that these proteins may have a role in the inductive interaction. The hypothesis that such proteins modulate the interaction must now be tested further by undertaking studies to determine their functional role. The approach that we have described here may also be useful for examining other inductive interactions to determine whether specific protein transfer is a common feature of such interactions and help clarify the signalling mechanisms in these systems.

We are grateful to Sandra Conlon for contributions critical to the completion of this work. CAP was supported by an NIH Training Grant. In addition, this work was supported by an NIH Research Career Development Award and grants from the American Cancer Society, NSF, and the Thomas F. and Kate Miller Jeffress Memorial Trust to RMG.

References

BONNER, W. M. & LASKEY, R. A. (1974). A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46**, 83–88.

BOUDET, A., HUMPHREY, T. J. & DAVIES, D. D. (1975). The measurement of protein turnover by density labeling. *Biochem. J.* **152**, 409–416.

DEMARCHEZ, M., MAUGER, A., HERBAGE, D. & SENGEL, P. (1984). Effect of hydrocortisone on skin development in the chick embryo: Ultra-structural, immunohistological and biochemical analysis. *Devl Biol.* **106**, 15–25.

DHOUAILLY, D., ROGERS, G. E. & SENGEL, P. (1978). The specification of feather and scale protein synthesis in epidermal–dermal recombinations. *Devl Biol.* **65**, 58–68.

FLICKINGER, R. A., HATTON, E. & ROUNDS, D. E. (1959). Protein transfer in chimaeric taricha-Rana explants. *Expl Cell Res.* **17**, 30–34.

FOURCROY, P. (1978). Advantages of potassium bromide gradients for isopycnic centrifugation of proteins. *Biochem. biophys. Res. Com.* **84**, 713–720.

GARRELS, J. I. (1979). Two-dimensional electrophoresis and computer analysis of proteins synthesized by clonal cell lines. *J. biol. Chem.* **254**, 7961–7977.

GOETINCK, P. F. & SEKELLICK, M. J. (1972). Observations on collagen synthesis, lattice formation, and morphology of scaleless and normal embryonic skin. *Devl Biol.* **28**, 636–648.

GRAINGER, R. M. & WESSELLS, N. K. (1974). Does RNA pass from mesenchyme to epithelium during an embryonic tissue interaction? *Proc. natn. Acad. Sci. U.S.A.* **71**, 4747–4751.

GROBSTEIN, C. (1957). Some transmission characteristics of the tubule-inducing influence on mouse metanephrogenic mesenchyme. *Expl Cell Res.* **13**, 575–587.

GROBSTEIN, C. (1959). Autoradiography of the interzone between tissues in inductive interaction. *J. exp. Zool.* **142**, 203–214.

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

KALLMAN, F. & GROBSTEIN, C. (1965). Source of collagen at the epitheliomesenchymal interfaces during inductive interaction. *Devl Biol.* **11**, 169–183.

KING, J. & LAEMMLI, U. K. (1971). Polypeptides of the tail fibers of bacteriophage T-4. *J. molec. Biol.* **62**, 465–473.

KITAMURA, K. (1981). Distribution of endogenous beta-galactoside-specific lectin, fibronectin, and type I and III collagens during dermal condensation in chick embryos. *J. Embryol. exp. Morph.* **65**, 41–56.

KOCH, W. E. & GROBSTEIN, C. (1963). Transmission of radioisotopically labeled materials during embryonic induction *in vitro*. *Devl Biol.* **7**, 303–323.

KOLLAR, E. J. (1983). Epithelial–mesenchymal interaction in the mammalian integument: Tooth development as a model for instructive induction. In *Epithelial–Mesenchymal Interactions in Development* (ed. R. H. Sawyer & J. F. Fallon), pp. 27–50. Praeger Publishers: New York.

LEFFAK, I. M., GRAINGER, R. M. & WEINTRAUB, H. (1977). Conservative assembly and segregation of nucleosomal histones. *Cell* **12**, 837–845.

MAUGER, A., DEMARCHEZ, M., HERBAGE, D., GRIMAUD, J. A.,

Druguet, M., Hartmann, D. & Sengel, P. (1982). Immunofluorescent localization of collagen types I and III, and of fibronectin during feather morphogenesis in the chick embryo. *Devl Biol.* **94**, 93–105.

Miller, E. J. (1984). Chemistry of the collagens and their distribution. In *Extracellular Matrix Biochemistry* (ed. K. A. Piez & A. H. Reddi), pp. 41–81. Elsevier Publishers.

Pantelouris, E. M. & Mulherkar, L. (1957). Tracer studies on chick embryos *in vitro*. *J. Embryol. exp. Morph.* **5**, 51–59.

Peterson, C. A. & Grainger, R. M. (1985). Differentiation of embryonic chick feather-forming and scale-forming tissues in transfilter cultures. *Devl Biol.* **111**, 8–25.

Peterson, C. A. & Grainger, R. M. (1986). Different protein synthetic patterns in scale-forming, feather-forming, and apteric embryonic chick dermis. *Devl Biol.* **114**, 102–108.

Phillips, W. H., Peterson, C. A. & Grainger, R. M. (1989). High resolution KSCN/CsSCN equilibrium gradients effectively separate a population of density labeled proteins from unlabeled proteins. *Anal. Biochem.* (in press).

Rawles, M. E. (1963). Tissue interactions in scale and feather development as studied in dermal-epidermal recombinations. *J. Embryol. exp. Morph.* **11**, 765–789.

Reddi, A. H. (1981). Cell biology and biochemistry of endochondral bone development. *Collagen Res.* **1**, 209–226.

Reddi, A. H. (1983). Role of extracellular matrix in cell differentiation and morphogenesis. In *Epithelial–Mesenchymal Interactions in Development* (ed. R. H. Sawyer & J. F. Fallon). pp. 75–92. New York: Praeger Publishers.

Salhanick, A. I., Krupp, M. N. & Amatruda, J. M. (1983). Dexamethosome stimulates insulin receptor synthesis in cultured rat hepatocytes. *J. biol. Chem.* **258**, 14 130–14 135.

Sampath, T. K., Nathanson, M. A. & Reddi, A. H. (1984). *In vitro* transformation of mesenchymal cells derived from embryonic muscle into cartilage in response to extracellular matrix components of bone. *Proc. natn. Acad. Sci. U.S.A.* **81**, 3419–3423.

Sampath, T. K. & Reddi, A. H. (1981). Dissociative extraction and reconstitution of extracellular matrix components involved in local bone differentiation. *Proc. natn. Acad. Sci. U.S.A.* **78**, 7599–7603.

Sirlin, J. L. & Brahma, S. K. (1959). Studies on embryonic induction using radioactive tracers. *Devl Biol.* **1**, 234–246.

Sirlin, J. L., Brahma, S. K. & Waddington, C. H. (1956). Studies on embryonic induction using radioactive tracers. *J. Embryol. exp. Morph.* **4**, 248–253.

Stuart, E. S., Garber, B. & Moscona, A. A. (1972). An analysis of feather germ formation in the embryo and *in vitro*, in normal development, and in skin treated with hydrocortisone. *J. exp. Zool.* **179**, 97–118.

Stuart, E. S. & Moscona, A. A. (1967). Embryonic morphogenesis: Role of fibrous lattice in the development of feathers and feather patterns. *Science* **157**, 947–948.

Vainio, T., Saxen, L., Toivonen, S. & Rapola, J. (1962). The transmission problem in primary embryonic induction. *Expl Cell Res.* **27**, 527–538.

(*Accepted 12 December 1988*)