Isolation and Preliminary Characterization of Epithelial-Specific Messenger Ribonucleic Acids and Their Products during Embryonic Tooth Development

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Experiments were designed to identify and characterize tissue-specific proteins involved in the process of tooth organogenesis. Epithelial and mesenchymal proteins were extracted from intact molar organs or mechanically separated tissues obtained from 25-day New Zealand White rabbit embryos. Labelling experiments with [³⁵S]methionine followed by radioautography or gel electrophoresis and fluorography showed the presence of label only in epithelial proteins. Most of these proteins range from 43 000 mol.wt. and higher, except for one band of approx. 16 000 mol.wt. A mRNA fraction of 16–26 S was isolated by ultracentrifugation on sucrose gradients. When translated in a reticulocyte-lysate cell-free system, the mRNA obtained from intact molar organs resulted in the synthesis of three proteins, of mol.wts. 65 000, 58 000 and 43 000. A similar mRNA fraction obtained from dental-pulp mesenchyme gave only the 43 000-mol.wt. protein, indicating that the 65 000- and 58 000-mol.wt. proteins are derived from epithelial cells.

Synthesis and secretion of characteristic proteins is an essential aspect of merocrine cell differentiation. Some well-known examples of such differentiation-specific protein synthesis are the production of ovalbumin by chick oviduct epithelium (Schimke *et al.*, 1972; Means & O'Malley, 1974; Towle *et al.*, 1976), amylase and insulin by pancreatic acinar epithelium (Rutter *et al.*, 1968; Rosen, 1976; Terry *et al.*, 1977; Rutter *et al.*, 1978), and type-II collagen by chondroblast cells and types-I and -III collagens by fibroblast cells (Gay & Miller, 1978). Particularly interesting are those factors controlling the synthetic rates of individual proteins during differentiation in epidermal organogenesis (e.g. pancreas, mammary gland, salivary gland and tooth organs) and the developmental programmes for sequential elaboration of several distinct gene products by a single cell type. The study of such processes in epidermal organ systems is often complicated by unavailability of synchronized cells at defined developmental stages, presence of extraneous cell types and concurrent mitotic activities. In this respect embryonic mammalian tooth organs, which consist of a single sheet of ectodermal-derived ameloblast cells adjacent to a sheet of cranial neural-crest-derived odontoblast cells, separated by their respective extracellular-matrix gene products, developmentally

synchronized and non-dividing, have certain advantages.

During embryonic tooth morphogenesis, odontoblast cell differentiation involves sequential elaboration of at least five different extracellular matrix proteins, types-I and -III collagens (Munksgaard *et al.*, 1978; Lesot *et al.*, 1978), proteoglycans (Engfeldt & Hjerpe, 1972; Linde, 1973), dentine phosphoproteins (Dimuzio & Veis, 1978; Butler *et al.*, 1979) and several other non-collagenous glycoproteins (Jones & Leaver, 1974). Epithelial ameloblast differentiation involves the production of at least a few enamel matrix proteins (i.e. 'amelogenins,' 'enamel proteins,' 'enameloids'). The dentine and enamel extracellular matrices therefore consist of several specific proteins, each produced during a characteristic developmental period (Fukae & Shimizu, 1974; Termine *et al.*, 1979; Slavkin *et al.*, 1979). The timing of synthesis and secretion of these merocrine-type-cell proteins is apparently controlled by a series of complex epithelial–mesenchymal interactions (Koch, 1967; Kollar, 1972; Slavkin, 1974; Slavkin *et al.*, 1979).

The present report focuses on ameloblast cell differentiation, the synthesis of unique epithelial-derived extracellular-matrix proteins and the isolation and characterization of their respective mRNA molecules. We have previously identified four extra-

Abbreviation used: SDS, sodium dodecyl sulphate.

cellular-matrix enamel proteins of 65 000, 58 000, 22 000 and 20 000 mol.wt. which characterize ameloblast differentiation in the embryonic New Zealand White rabbit (Guenther *et al.*, 1977). We have also reported preliminary evidence supporting the feasibility of isolating biologically active mRNA species from embryonic-rabbit molar tooth organs (Lee-Own *et al.*, 1977). We now describe solubilization and partial characterization of the tooth epithelial-derived proteins, which are distinct from the odontoblast-derived dentine matrix proteins. We present evidence that several ameloblast-cell-differentiation proteins are synthesized as distinct gene products. We report isolation of epithelial-specific mRNA molecules ranging from 16 to 26S which characterize ameloblast cells.

Finally, we demonstrate that an active system for cell-free protein biosynthesis using isolated epithelial-specific mRNA species will produce gene products equivalent to ameloblast proteins produced *in vivo*.

Materials and Methods

All the reagents used were the best grade available. Proteinase K was obtained from Beckman Instruments, Fullerton, CA, U.S.A.; heparin was from Sigma Chemical Co., St. Louis, MO, U.S.A.; phenol (distilled before use) and formamide were obtained from Fisher Scientific Co., Fairlawn, NJ, U.S.A.; oligo(dT)-cellulose (type III) was from Collaborative Research, Waltham, MA, U.S.A.; acrylamide, bisacrylamide, *NNN'*-tetramethylethylenediamine, ammonium persulphate, SDS and the reagents for protein determination by the Bradford (1976) method were from Bio-Rad, Richmond, CA, U.S.A.; ultrapure sucrose (ribonuclease-free), Tris and urea were from Schwartz/Mann, Orangeburg, NY, U.S.A.; molecular-weight protein standards were from Pharmacia Fine Chemicals, Uppsala, Sweden. [³⁵S]Methionine (829.29 Ci/mmol specific radioactivity), reticulocyte-lysate 'Translation Kit' and Aquasol were from New England Nuclear Corp., Boston, MA, U.S.A. The radiolabelled purified amylase used as molecular-weight standard for fluorography (55 000 mol.wt) was a kind gift from Dr. Barry Feinberg, University of Southern California Dental School. All the solutions were kept under maximum sterile conditions and all the glassware was either disposable or autoclaved.

Tooth-organ dissection

Embryonic tooth organs were obtained from 25-day New Zealand White rabbit embryos. At this developmental stage, both mesenchymal and epithelial cell-derived extracellular-matrix gene products are being synthesized and secreted (Slavkin *et al.*, 1978). The first and second molar organs were carefully dissected in order not to disrupt the epi-

thelial cell layer, and immediately placed in cold 0.1 M-sodium phosphate/0.9% NaCl buffer containing a proteinase inhibitor (1 mM-phenylmethanesulphonyl fluoride). The enamel-organ epithelium was separated from the dental-pulp mesenchyme when the experimental protocol so required.

For the RNA extraction, the intact molars were frozen immediately after dissection by placing them in a sterile plastic tube kept in solid CO₂, and then they were stored at -90°C. Repeated dissections were performed and the dissection specimens were kept at -90°C until adequate sample sizes were collected to proceed with the subsequent RNA studies.

Protein extraction

The intact molar organs, the isolated enamel-organ epithelia and dental-pulp mesenchyme were rinsed several times with 0.1 M-phosphate/saline buffer containing 1 mM-phenylmethanesulphonyl fluoride. The proteins in each sample were then extracted with 40 µl of homogenization buffer (containing 125 mM-Tris/HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 250 mM-sucrose and 0.01% Bromophenol Blue)/ten teeth (Patzelt *et al.*, 1978). The samples were boiled for 5 min and centrifuged at 13 000g for 10 min. The supernatant containing the proteins extracted was carefully removed and immediately applied to polyacrylamide gels.

Radioactively labelled proteins

The intact molar organs were incubated in Eagle's Minimum Essential Medium (Gibco, Grand Island, NY, U.S.A.) containing 25 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.4, in the presence of 50 µCi of [³⁵S]methionine/ml for 4 h at 37°C. The incubation was terminated by removing the medium and replacing it with fresh medium containing an excess of non-radioactive methionine and maintained at 4°C. The enamel-organ epithelium was physically separated from the dental-pulp mesenchyme at this point, and each sample was then extracted as described above.

Radioautography

After incubation with the radioactive amino acid precursor, the intact molar organs were washed with 5 × 1 ml of 0.1 M-phosphate/saline buffer, fixed in 10% neutral buffered formalin (Lerner Laboratories, Stamford, CT, U.S.A.) and processed for light-microscopic radioautography. After dehydration, the specimens were embedded in JB-4 methacrylate (Polysciences, Warrington, PA, U.S.A.) and 1.5 µm-thick sections were prepared.

Protein determination

The method described by Bradford (1976) was used.

Polyacrylamide-gel electrophoresis

Slab gels containing 10% (w/v) acrylamide were used for the analysis of the gene products, as described by Davis (1964) and Ornstein (1964), with the following modifications. SDS (0.1%) was present in both the gel and the running buffer, and the gel also contained 1 M-urea. The gels were run at 3 mA/gel for 17–20 h, stained with Coomassie Blue, destained and prepared for fluorography as described by Bonner & Laskey (1974). The dry gels were exposed to pre-flashed RP Royal X-omat X-ray film (Laskey & Mills, 1975). The molecular weights of the proteins were determined by their relative mobilities compared with molecular-weight standards (Dunker & Rueckert, 1969).

Total RNA extraction

Approx. 1600 molar organs dissected from 200 embryos were used per experiment (about eight or ten embryos per litter). The RNA was extracted by the proteinase-K method (Rowe *et al.*, 1978). For each 1 g of frozen organ or tissue, 10 ml of the following buffer was added: 10 mM-Tris/HCl, pH 7.5, 1% SDS, 5 mM-EDTA, 65 µg of proteinase K/ml. The samples were homogenized at room temperature with a Tissumizer (Tekmar Co., Cincinnati, OH, U.S.A.) and incubated for 1 h at 37°C. The homogenate was adjusted to 1% Triton X-100, 1% sodium deoxycholate, 0.1 M-NaCl and 0.5 mg of heparin/ml. The RNA was extracted three times with phenol/chloroform (1:1, v/v), precipitated with ethanol and washed several times (Rowe *et al.*, 1978). The concentration of RNA was measured by the A_{260} of the aqueous RNA solution by assuming that 1 A_{260} unit = 50 µg of RNA. The RNA was kept precipitated at -20°C until used.

Isolation of poly(A)-containing RNA

The total RNA was passed through an oligo(dT)-cellulose column as described by Aviv & Leder (1972). Each of the RNA fractions eluted from the column was precipitated with ethanol and kept at -20°C until further utilized.

Fractionation of poly(A)-containing RNA

This was accomplished by ultracentrifugation on sucrose gradients containing 50% (v/v) formamide as previously described (Zeichner & Stern, 1977). Linear gradients from 5 to 20% (w/v) sucrose in 0.1 M-LiCl/0.05 M-EDTA/0.2% SDS/0.01 M-Tris/HCl, pH 7.4, were loaded with 350 µg of heat-denatured RNA/ml. The gradients were centrifuged at 25°C for 16 h at 30 000 rev./min in a Beckman L2-65B ultracentrifuge by using a SW-40 rotor. Fractions (0.4 ml) were collected and the A_{254} was continuously monitored. The fractions were pooled, precipitated with ethanol, washed several times with

0.4 M-NaCl/66% (v/v) ethanol to eliminate the formamide, and kept at -90°C.

Determination of translational activity

The cell-free system used was the reticulocyte-lysate 'Translation Kit' prepared by New England Nuclear Corp., Boston, MA, U.S.A., with [35 S]-methionine. The method described by the suppliers was followed. The reactions were carried in 25 µl assay mixtures containing 22 µCi of [35 S]-methionine, 2 mM-magnesium acetate, 80 mM-potassium acetate and 1–10 µg of RNA. The samples were incubated for 1 h at 37°C. Samples (1 µl) were taken to determine the radioactivity incorporated by placing them on a glass-fibre filter, followed by precipitation with cold 10% (v/v) trichloroacetic acid. After several washes with trichloroacetic acid, the filters were boiled for 10 min, cooled, washed again with 5% trichloroacetic acid, rinsed with ethanol, and air-dried. The filters were counted for radioactivity by conventional methods of spectrometry after the addition of 5 ml of Aquasol. The rest of the sample was treated with an equal volume of polyacrylamide-gel sample buffer, reduced with mercaptoethanol, heat-denatured at 70°C for 15 min and analysed by polyacrylamide-gel electrophoresis and fluorography.

Results

Analysis of gene products

In order to discriminate between the proteins derived from the mesenchymal cells and those derived from the epithelial cells, the dental-pulp mesenchyme was physically separated from the enamel-organ epithelium (containing the epithelial cell layer and extracellular matrix). The proteins were extracted from each sample as described in the Materials and Methods section and then fractionated and analysed by polyacrylamide-gel electrophoresis. The densitometric profiles obtained from the stained gels are shown in Fig. 1. Although it might seem that all three profiles are very similar, careful analysis shows some qualitative and quantitative differences. There were at least six protein bands identified in the intact molar sample, which were also present in the enamel-organ epithelium sample and apparently absent from the dental-pulp mesenchyme sample. These proteins were identified as A, B, C, D, E and F (as shown in Fig. 1), and their absence from the dental-pulp mesenchyme might suggest an epithelial origin. Also, the relative proportions between bands were different from one tissue to another.

Analysis of newly synthesized proteins

To discriminate between proteins that accumulated within these tissues during previous development, and the newly synthesized and sec-

Fig. 1. Comparison of proteins extracted from molar tooth organs and their constituent tissues

Proteins were extracted from intact molars (IM) or their mechanically separated tissues [enamel-organ epithelia (EOE) and dental-pulp mesenchyme (DPM)] and analysed by gel electrophoresis in 10% acrylamide slab gels as described in the Materials and Methods section. Gels were stained with Coomassie Blue and the densitometric profiles were obtained with a Helena Quick-scan Densitometer (Helena Laboratories, Beaumont, TX, U.S.A.). The molecular weights for the proteins indicated were determined by their mobilities relative to molecular-weight standards: A, 72 000; B, 65 000; C, 58 000; D, 43 000; E and F, approx. 16 000.

reted extracellular-matrix proteins, intact molar organs were routinely incubated with [^{35}S]methionine and cultured for 4 h *in vitro*. [^3H]Proline and [^3H]leucine were also found to be effective precursors in comparable studies. The incorporation of the labelled amino acid was followed by light-microscopic high-resolution radioautography. After a 4 h labelling period, the silver grains were localized mainly over the secretory ameloblast cells and over the enamel extracellular matrix (Fig. 2). Under these conditions, very few silver grains were found over the odontoblast cells or over the extracellular dentine matrix. These results indicate that all the proteins labelled with the radioactive amino acid precursor were newly synthesized and the secreted products were derived from cells within the enamel-organ epi-

Fig. 2. Incorporation of [^{35}S]methionine into New Zealand White rabbit embryo molar tooth organ

The distribution of silver grains after 4 h incubation of molar tooth organs with [^{35}S]methionine was analysed by light-microscopy radioautography. The enamel-organ epithelia (EOE) contains the secretory ameloblast cells (Am) and the enamel extracellular matrix (indicated by the arrows). The dental-pulp mesenchyme (DPM) contains the secretory odontoblast cells (Od).

thelia, specifically from cells within the inner enamel epithelium (e.g. pre-ameloblast and ameloblast cells).

The newly synthesized and secreted proteins were extracted from both intact molars and enamel-organ epithelia. These labelled epithelial proteins were fractionated by polyacrylamide-gel electrophoresis and analysed by fluorography. Since no radioactivity was detected in the proteins extracted from the dental-pulp mesenchyme samples, and the labelling pattern obtained for the enamel-organ epithelia was identical with the one obtained for the intact molars, only the results corresponding to the last sample are shown in Fig. 3. The fluorography pattern resembled very closely the stained gel, indicating that most of the proteins present were newly synthesized. Nevertheless, it is very important to note that most of the labelled proteins were of relative high molecular weights (from 43 000 up), except for one heavy labelled protein of approx. 16 000 mol.wt. These results indicate that the proteins of mol.wt. lower than 43 000 (except that of 16 000 mol.wt.) were not synthesized during the 4 h period of culture with the amino acid precursor.

Isolation of mRNA species

Intact molars were used to obtain the RNA in order to avoid a prolonged period of tissue manipulation, which might increase RNA degradation by

nucleases. RNA from isolated dental-pulp mesenchyme was extracted to differentiate between the mRNA species present in this mesenchyme tissue and those found within the enamel-organ epithelia. The presence of mRNA molecules during the purification process was monitored by their translational activity in a cell-free system derived from reticulocyte lysate. The total RNA extracted from intact molars and isolated dental-pulp mesenchyme was separated into poly(A)-containing RNA

(mRNA) and non-poly(A)-containing RNA (rRNA and tRNA) by chromatography on oligo(dT)-cellulose. An average yield of 5–6 mg of total RNA was obtained from approx. 1600 molar organs or dental-pulp mesenchyme. Of the total RNA, 6% was bound to the oligo(dT)-cellulose.

The poly(A)-containing RNA species (i.e. 6% of the total RNA) were further fractionated according to their sedimentation coefficients by ultracentrifugation in sucrose gradients under denaturation conditions. The mRNA fraction sedimenting in the 16–26S region was pooled and tested for its translational activity in the cell-free system. The enrichment of mRNA activity throughout the purification steps is shown in Table 1.

Fig. 3. Gel-electrophoretic analysis of newly synthesized proteins extracted from intact molar tooth organs

Intact molar tooth organs were incubated for 4 h with [35 S]methionine, the proteins extracted and analysed in 10% acrylamide slab gels as described in the Materials and Methods section. The gels were stained (2), prepared for fluorography and exposed to RP Royal X-omat X-ray film (3). Molecular-weight standards were run in the same gel (1) and 14 C-labelled amylase purified from salivary glands was used as fluorography molecular-weight marker (4).

Characterization of epithelial-specific mRNA species

The translational products obtained in the cell-free system with the 16–26S mRNA fraction from intact molar organs or dental-pulp mesenchyme were analysed by polyacrylamide-gel electrophoresis followed by fluorography. The results presented in Fig. 4 showed that the reticulocyte-lysate cell-free system alone, with no exogenous mRNA added, synthesized two proteins. Their molecular weights were determined as 49 000 and 19 000 respectively. The presence of the 16–26S mRNA fraction from the intact molars showed that, in addition to these proteins, two distinctively different proteins of higher molecular weight were also present. These two proteins have mol.wts. of 65 000 and 58 000 respectively. Also, the protein band of 49 000 mol.wt. was broader than that present in the control, indicating the possibility of another protein being synthesized in the presence of the exogenous mRNA which migrates in the same position. When the 16–26S mRNA fraction obtained from the dental-pulp mesenchyme was used as template, neither the 65 000- nor the 58 000-mol.wt. gene product was detected. Nevertheless, a similar broad band in the 49 000-mol.wt. region was present. These results clearly indicate that the 65 000- and 58 000-mol.wt. proteins synthesized in the presence of a 16–26S mRNA from intact molars are derived from epithelial cells, whereas the other protein (approx.

Table 1. Enrichment of mRNA translational activity through purification

The specific activity was determined as c.p.m. of [35 S]methionine incorporated into trichloroacetic acid-precipitable protein/ μ g of RNA added to the reaction mixture.

Sample	Intact molars		Dental-pulp mesenchyme	
	Specific activity	Enrichment	Specific activity	Enrichment
Total RNA	19 521	1	10 732	1
Poly(A)-containing RNA	130 166	6.6	138 353	12.8
16–26S mRNA	244 037	13	278 011	25.8

Fig. 4. Gel-electrophoretic analysis of products of mRNA translation in the cell-free system

Fluorographs of 10% polyacrylamide slab gels containing the reticulocyte-lysate cell-free system products when: no exogenous mRNA was added (2), 2 μ g of intact molar 16–26S mRNA was added (4) and 2 μ g of dental-pulp mesenchyme 16–26S mRNA was added (3). 14 C-labelled amylase (55 000 mol.wt.) was used as molecular-weight marker (1).

Fig. 5. Relationship between mRNA species and proteins present in the rabbit tooth organ

Comparison of the cell-free-system translational products obtained with the 16–26S mRNA (3) and the newly synthesized proteins (4) extracted from intact molars shows the correlation between these gene products. The characterization of the proteins is facilitated by including the molecular-weight marker (1) and the reticulocyte-lysate endogenous cell-free product (2) in the Figure.

mol.wt. 49 000) could be derived from mesenchymal cells.

Identification of the mRNA cell-free-system products

To establish an identity between the proteins synthesized from the isolated mRNA species and the proteins that were synthesized in the intact molar organ culture, the fluorography profiles obtained for each one were compared as shown in Fig. 5. All three proteins synthesized in the cell-free system correspond to an equivalent protein present in the original tissue. The 16–26S mRNA fraction contains the information for three proteins, of which two are definitely epithelial-specific gene products (65 000 and 58 000 mol.wts.), whereas the third (determined as 43 000 mol.wt.) could be a non-specific gene product from both epithelial and mesenchymal cells.

Discussion

The immediate objective of these studies was to identify specific epithelial gene products and their

respective mRNA species involved in the process of embryonic tooth organogenesis. Our long-term objective is to understand the process of epithelial cell differentiation and the regulation of gene expression during organogenesis. With these objectives in focus, we designed our experimental strategy as follows: (a) selection of a suitable tooth organ in relation to its specific developmental stage; (b) analysis of how many proteins comprise the unique phenotype of the tooth organ; (c) localization of the distribution of labelled amino acid precursor incorporated into tissue-specific gene products during the selected developmental stage of tooth morphogenesis; (d) determination of which phenotypically characteristic proteins are synthesized at this stage of development; (e) determination of which of these newly synthesized proteins are derived from which specific tissue; (f) isolation of fractions which contain mRNA transcripts unique for specific tissue-derived proteins; (g) obtaining the translational gene products from these tissue-specific mRNA fractions; and (h) establishment of the relationship between the mRNA-translational products and the tissue-specific newly synthesized proteins.

For these studies, the judicious selection of organ developmental stage is imperative. We selected the molar tooth organ from 25-day New Zealand White rabbit embryos because at this developmental stage the odontogenic organ contains differentiated secretory odontoblast and ameloblast cells which synthesize and secrete extracellular-matrix gene products. At this developmental stage the organs also contain less differentiated mesenchymal and epithelial cells, which we have assumed are actively engaged in the synthesis of tissue-specific mRNA molecules before dentine and enamel production.

To characterize tissue-specific gene products, those that best resembled the nascent translated gene products, we have designed our studies to isolate the recently synthesized proteins contained in the enamel-organ epithelia from organ cultures. The intact molar organs were labelled with [35 S]methionine, and then analysed as such or mechanically separated into mesenchymal or epithelial components. Under these incubation conditions, only the secretory ameloblast-cell layer and the forming enamel matrix were labelled (Fig. 2). Although the odontoblasts were also actively synthesizing proteins, very few silver grains resulting from light-microscopic radioautography were localized either over the odontoblast cells or over the dentine matrix. The absence of silver grains over the odontoblast cells or dentine matrix was possibly the result of a physical barrier from the surrounding epithelia which did not allow diffusion of the [35 S]methionine into the dental-pulp mesenchyme. This observation was extremely fortuitous; we could use the intact molar organ for culture studies *in vitro*, having confidence that the newly synthesized isotopically labelled proteins were epithelial-specific gene products. This conclusion was proved to be valid; for example, when the fluorographs obtained from intact molar organs or isolated enamel-organ-epithelial samples were critically compared, they were identical.

Although many of the proteins present in the molar organs were labelled with the amino acid precursor, most labelled proteins were of relatively high mol.wt. (43 000 and higher). Only one protein of lower mol.wt. (approx. 16 000) was routinely labelled. In contrast, the Coomassie-Blue-stained gels, representing the total number of extracted proteins, also contained several low-molecular-weight proteins assumed to be derived from larger proteins.

Secreted extracellular-matrix proteins are often synthesized in a precursor form, which is subsequently processed into modified forms by sequential enzymic cleavage, post-translational processing, during intracellular transport and secretion. Using the paradigm preprocollagen $\rightarrow$ procollagen $\rightarrow$ collagen, we have assumed that 'preproenamel' is also post-translationally processed into increasingly smaller polypeptides concomitant with increased

extracellular-matrix-enamel mineralization (Slavkin *et al.*, 1978; Chrispens *et al.*, 1979). After secretion from the ameloblasts, the enamel proteins are further processed by an enamel peptidase (Suga, 1970; Shimizu *et al.*, 1979). The fact that most of the newly synthesized proteins present were of high molecular weight favours the concept of a precursor(s) for enamel proteins.

Our results indicated that there were at least six proteins (A, B, C, D, E and F) possibly derived from epithelial cells; all six polypeptides were routinely labelled *in vitro* (Fig. 3). Two of these proteins (E and F) were of low molecular weight, whereas proteins A, B, C and D ranged from 72 000 to 43 000 mol.wt. (Fig. 4). These results agree with previous reports from our laboratory (Guenther *et al.*, 1977) and other laboratories (Termine *et al.*, 1979), which indicated that the 'enamel proteins' were three or four proteins with mol.wts. from 72 000 to 20 000. However, it still remains to be determined if all of these proteins are nascent gene products. To address this question, we isolated a mRNA fraction large enough to contain mRNA species that could code for any of these proteins. Assuming that the epithelial-derived gene products were in a range from 72 000 to 43 000 mol.wt., we selected mRNA molecules sedimenting between 16S and 26S (assuming that a protein of 43 000 mol.wt. needs 1290 bases for coding and a protein of 72 000 mol.wt. requires 2100 bases; all these mRNA species are contained in these fractions). The translated mRNA fraction in a cell-free system produced only three of these proteins, those of mol.wts. 65 000, 58 000 and 43 000, which corresponded to proteins B, C and D respectively. Proteins B and C were definitely epithelial-specific proteins, as established by the fact that the dental-pulp mesenchyme does not contain these specific mRNA species. The origin of protein D (43 000 mol.wt.) was more difficult to establish, since both epithelial and mesenchymal mRNA species synthesized a protein having this relative electrophoretic mobility on SDS/polyacrylamide slab gels (Fig. 5).

At present, we cannot discard the possibility that the lower-molecular-weight proteins (E and F) are also nascent gene products. Lower-sedimentation-coefficient mRNA fractions should be tested in the cell-free system to obtain this information. Also, further characterization of the proteins extracted from the organ cultures and the proteins synthesized in the cell-free system by using amino acid composition and peptide 'maps' should be done.

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References

- Aviv, H. & Leder, P. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1408–1412
- Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88
- Bradford, M. M. (1976) *Anal. Biochem.* **12**, 248–254
- Butler, W. T., Munksgaard, E. C. & Richardson, W. S. (1979) *J. Dent. Res.* **58**, 817–824
- Chrispens, J., Weliky, B., Bringas, P. & Slavkin, H. C. (1979) *J. Dent. Res.* **58**, 988–990
- Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
- Dimuzio, M. T. & Veis, A. (1978) *Calcif. Tissue Res.* **25**, 169–178
- Dunker, A. K. & Rueckert, R. R. (1969) *J. Biol. Chem.* **244**, 5074–5080
- Engfeldt, B. & Hjerpe, A. (1972) *Calcif. Tissue Res.* **10**, 152–159
- Fukae, M. & Shimizu, M. (1974) *Arch. Oral Biol.* **19**, 381–386
- Gay, S. & Miller, E. J. (1978) *Collagen in the Physiology and Pathology of Connective Tissue*, Gustav Fischer Verlag, Stuttgart and New York
- Guenther, H. L., Croissant, R. D., Schonfeld, S. E. & Slavkin, H. C. (1977) *Biochem. J.* **163**, 591–603
- Jones, I. L. & Leaver, A. G. (1974) *Arch. Oral Biol.* **19**, 371–380
- Koch, W. E. (1967) *J. Exp. Zool.* **165**, 155–170
- Kollar, E. J. (1972) in *Developmental Aspects of Oral Biology* (Slavkin, H. C. & Bavetta, L. A., eds.), pp. 125–149, Academic Press, New York
- Laskey, M. A. & Mills, A. D. (1975) *Eur. J. Biochem.* **56**, 335–341
- Lee-Own, V., Zeichner, M., Benveniste, K., Denny, P., Paglia, L. & Slavkin, H. C. (1977) *Biochem. Biophys. Res. Commun.* **74**, 849–856
- Lesot, H., Von der Mark, K. & Ruch, J. V. (1978) *C.R. Hebd. Seances Acad. Sci. Ser. D* **286**, 765–768
- Linde, A. (1973) *Calcif. Tissue Res.* **12**, 281–294
- Means, A. R. & O'Malley, B. W. (1974) in *Biochemistry of Cell Differentiation* (Paul, J., ed.), vol. 9, pp. 161–180, University Park Press, Baltimore
- Munksgaard, E. C., Rhodes, M., Mayne, R. & Butler, W. T. (1978) *Eur. J. Biochem.* **82**, 609–617
- Ornstein, L. (1964) *Ann. N.Y. Acad. Sci.* **121**, 321–348
- Patzelt, C., Labrecque, A. D., Dugid, J. R., Carroll, R. J., Keim, P. S., Heinrikson, R. L. & Steiner, D. F. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1260–1264
- Rosen, J. M. (1976) *Biochemistry* **15**, 5263–5271
- Rowe, D. W., Moen, R. C., Davidson, J. M., Byers, P. M., Bornstein, P. & Palmiter, R. (1978) *Biochemistry* **17**, 1581–1590
- Rutter, W. J., Clark, W. R., Kemp, J. D., Bradshaw, W. S., Sanders, T. G. & Ball, W. D. (1968) in *Epithelial-Mesenchymal Interactions* (Fleischmajer, R. & Billingham, R. E., eds.), pp. 114–131, Williams and Wilkins, Baltimore
- Rutter, W. J., Pictet, R. L., Harding, J. D., Chivgwin, J. M., McDonald, R. J. & Brzybyla, A. E. (1978) in *Molecular Control of Proliferation and Differentiation* (Papaconstantinou, J. & Rutter, W. J., eds.), pp. 205–227, Academic Press, New York
- Schimke, R. T., Palmiter, R. D., Palacios, R., Rhoades, R. E., McKnight, S., Sullivan, D. & Summers, M. (1972) in *Genetic Mechanisms of Development* (Ruddle, F. H., ed.), pp. 225–249, Academic Press, New York
- Shimizu, M., Tanabe, T. & Fukae, M. (1979) *J. Dent. Res.* **58**, 782–789
- Slavkin, H. C. (1974) in *Oral Sciences Reviews* (Melcher, A. H. & Zarb, G. A., eds.), vol. 4, pp. 28–33, Munksgaard, Copenhagen
- Slavkin, H. C., Trump, G. N., Schonfeld, S. E., Brownell, A. G., Sorgente, N. & Lee-Own, V. (1978) in *Cell Interactions in Differentiation* (Saxen, L. & Weiss, L., eds.), pp. 209–226, Academic Press, New York
- Slavkin, H. C., Weliky, B., Stellar, W., Slavkin, M. D., Zeichner-Gancz, M., Bringas, P., Hyatt-Fischer, H., Shimizu, M. & Fauke, M. (1979) *J. Biol. Buccale* **309–326**
- Suga, S. (1970) *Arch. Oral Biol.* **15**, 555–558
- Termine, J. D., Torchia, D. A. & Conn, K. M. (1979) *J. Dent. Res.* **58**, 773–778
- Terry, P. M., Banerjee, M. R. & Lui, R. M. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2441–2445
- Towle, H. C., Tsai, M. J., Hirose, M., Tsai, S. Y., Schwartz, R. J., Parker, M. G. & O'Malley, B. W. (1976) in *The Molecular Biology of Hormone Action* (Papconstantinou, J., ed.), pp. 107–136, Academic Press, New York
- Zeichner, M. & Stern, R. (1977) *Biochemistry* **16**, 1378–1382