CELL-FREE TRANSLATION OF MESSENGER RNAs OF EMBRYONIC TOOTH ORGANS: SYNTHESIS OF THE MAJOR EXTRACELLULAR MATRIX PROTEINS

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Summary: In order to understand the regulation of embryonic mammalian enamel and dentine extracellular matrix protein synthesis, the biological activity of embryonic rabbit molar tooth organ messenger RNAs has been examined. Total RNA was extracted from 26-day embryonic tooth organs and fractionated by chromatography on oligo(dT)-cellulose. Replicate samples were fractionated on sucrose density gradients and the poly(A)-containing distribution determined using a poly(U) ^{3}H assay. The poly(A)-containing fractions stimulated ^{3}H-proline incorporation 10-fold in wheat germ cell-free extracts. Analysis of the labelled reaction products on sodium dodecyl sulphate-polyacrylamide gels revealed seven major peaks, one co-migrating with procollagen alpha chains (circa 145,000 daltons) and the others migrating slightly faster than the various extracellular matrix proteins which characterize amelogenesis and dentinogenesis. Purified collagenase digestion of the cell-free reaction products eliminated the 145,000 dalton procollagen-like polypeptide. This is the first demonstration of the isolation of embryonic tooth organ messenger RNAs and provides an experimental approach by which to study the regulation of extracellular matrix formation during tooth morphogenesis. We predict that the non-collagenous proteins synthesized *in vitro* represent enamel proteins, alkaline phosphatase, dentine phosphoproteins and proteins associated with proteoglycans.

INTRODUCTION

The regulator processes operating during embryonic epithelial-mesenchymal interactions associated with tooth organ morphogenesis are as yet obscure! The value of the tooth organ system lies in its high degree of developmental programming for sequential synthesis of many extracellular matrix proteins derived from either the epithelium or the adjacent mesenchyme (e.g. collagens,

enamel proteins, alkaline phosphatase, dentine phosphoproteins, and proteoglycans) (2). Substantial progress has been made in the isolation and purification of single mRNA species from cells highly differentiated for the synthesis of one or a few proteins (3). In contrast, cell differentiation and histogenesis during embryonic epidermal organ formation (e.g. tooth development, salivary gland, pancreas, mammary gland) often entails a precise program of changing protein synthetic patterns.

Our laboratory has assumed that understanding the biochemical basis underlying the formation of the extracellular matrix would be facilitated by the isolation of mRNAs present during overt embryonic tooth organogenesis. In this report we will show that by combining a sensitive cell-free translation system from wheat-germ extracts (4) with relatively simple methods of purification of embryonic tooth organ messenger RNAs (mRNAs), based on the presence of polyadenylate poly(A) sequences (5, 6), it is possible to synthesize and identify the major extracellular matrix proteins characteristic of dentine and enamel matrix formation (7).

MATERIALS AND METHODS

Isolation of Total Cellular mRNAs. All solutions and glassware were sterilized by autoclaving. In each experiment, 160-200 molar tooth organs were dissected from four litters of 26-day embryonic New Zealand White rabbits (7). At this stage of tooth morphogenesis both dentine and enamel extracellular matrix formation are major metabolic activities (7). The organs were pulverized and homogenized using a mortar and pestle in 5 volumes of 0.1 M Tris-acetate, pH 9.0, 0.1 M NaCl, 2 mM Na_2 EDTA, 0.5% sodium dodecyl sulphate, 100 µg/ml polyvinyl sulphate, and 2 mg/ml bentonite. The extraction buffer was shaken vigorously before an equal volume of chloroform-phenol was added (5). The aqueous phase was separated and re-extracted twice with chloroform-phenol. The final aqueous phase was made 2% with potassium acetate, pH 5.5, and the RNA was precipitated with 2.5 volumes of ethanol at 20°C. The RNA pellet was washed with 3 M sodium acetate, pH 6.5, and finally with 70% ethanol in 0.1 M sodium acetate. The RNA preparation was dried and dissolved in 0.4 M NaCl, 10 mM Tris-acetate, pH 7.6, 0.5% SDS, and fractionated on either a 5-20% sucrose gradient, or fractionated on an oligo(dT)-cellulose column. RNA concentration was determined from absorbance at 260 nm assuming an absorbance of 20 is equivalent to 1 mg of RNA. The A_{260}/A_{280} ratio was generally 1.8. This general procedure usually gave a yield of 540-580 µg total RNAs per 4 litters of embryonic tooth molars. From this RNA preparation we usually obtained 8-10 µg total poly(A)-containing RNAs.

Preparation of Wheat Germ Cell-free Extracts. Uncooked wheat germ was used for making cell-free extracts as described by Roberts and Paterson (4) except that the grinding buffer contained 1 mM dithiothreitol and the G-25

chromatography buffer contained 0.5 mM dithiothreitol. This substitution gave extracts with superior activity.

Identification of poly(A) in RNA fractions. The presence of poly(A) in RNA fractions from sucrose density gradients was tested by hybridization with an excess of labeled poly(U). The reaction mixtures contained, in a final volume of 0.5 ml: an aliquot of RNA, 5 µCi of (^{3}H)poly(U), 50 mM Tris-acetate, pH 7.4, 0.2 M NaCl, and 5 mM magnesium acetate. Incubation was for 15 min. at 25°C, after which boiled pancreatic ribonuclease (20 µg/ml) was added and incubated for an additional 30 min. The reaction was terminated by adding cold 5% trichloroacetic acid with 100 µg of yeast RNA as carrier. The precipitate was collected on Whatman filter paper, washed with cold 5% TCA, dried, and the radioactivity was measured in a Beckman scintillation counter.

Translation of Tooth Organ mRNAs in Wheat Embryo Cell-free System. Poly(A)-containing RNAs were translated in an amino acid incorporation system from wheat. The optimal ionic conditions for the translation of embryonic tooth organ RNAs indicated potassium and magnesium optima of 80 mM and 3 mM, respectively (8-10). Similar results were obtained when protein synthesis was directed by a control messenger RNA (globin mRNA) obtained as a gift from Dr. Philip Leder (National Institute for Child Growth and Development, National Institutes of Health, Behtesda, Maryland).

Sodium Dodecyl Sulphate Gel Electrophoresis. Labeled and unlabeled extracellular matrix protein extracts were subjected to electrophoresis on either 5% or 7.5% SDS gels as previously described (7). Molecular weights were determined by running purified proteins of known molecular weights on parallel gels. The standards used were gamma, beta and alpha chains of type I rat skin collagen, bovine serum albumin (67,500), ovalbumin (45,000), chymotrypsin A (25,000) and ribonuclease A (13,700). The isotopically-labeled reaction mixtures resulting from the *in vitro* cell-free protein synthesis system were solubilized in SDS buffer and run on duplicate gels and compared with the electrophoretic mobilities of extracellular matrix proteins (e.g. collagens, enamel proteins, alkaline phosphatase, dentine phosphoproteins and proteoglycans). Gels were routinely analyzed by cutting gels into 1 mm slices and the radioactivity was measured in a liquid scintillation spectrometer.

RESULTS AND DISCUSSION

The rapid rise in extracellular matrix protein number and concentration during late embryonic mammalian tooth development *in vivo* could be the result of *de novo* synthesis of translatable mRNAs for procollagen polypeptide chains (circa 145,000 daltons), dentine phosphoproteins, alkaline phosphatase, enamel proteins and proteoglycans. Accordingly, total cellular RNA was isolated from whole tissues (including polysomal and non-polysomal RNA) and translated in the wheat embryo cell-free system. Further studies determined the stimulation of amino acid incorporation in wheat germ cell-free extracts by various fractions obtained after chromatography of total molar tooth organ RNA on oligo(dT)-cellulose. Amino acid incorporation was significantly stimulated

TABLE I

CHARACTERISTICS OF THE WHEAT EMBRYO CELL-FREE PROTEIN SYNTHESIZING SYSTEM DIRECTED BY EXOGENOUS mRNAs

Reaction Components	Incorporation of ^{3}H proline for 30 min. at 30°C (cpm)
Complete System	
Globin RNA as messenger (2 µg)	16,000
Globin RNA as messenger (5 µg)	25,400
Poly(A)-containing tooth RNA (1 µg)	8,000
Poly(A)-containing tooth RNA (5 µg)	23,600
Deletions	
Messenger RNA	1,600
Wheat ribosomes	300
Supernatant factors	180
Energy-generating system	81

The yield of poly(A)-containing RNA from the 26-day embryonic New Zealand White rabbit molar tooth organs was 1-2%. Generally, the yield of total RNA was 540-580 µg per four litters of embryos representing 160-200 individually dissected tooth organs. Routine protein synthesis assays contained 3mM magnesium ions, 80-150 mM potassium ions, 5 µg RNA and 30 µCi ^{3}H-proline incubated for 60 min. in a total 100 µl reaction volume.

by the RNA fractions that bind to oligo(dT)-cellulose at high ionic strength. This is consistent with RNA fractions containing poly(A) sequences as shown for numerous eucaryotic mRNAs (3) (Table 1).

The stimulation of isotopic proline into TCA-precipitable reaction products varied from experiment to experiment; stimulation varied from as little as 4-fold to 10-fold increases in the wheat germ extracts. In experiments in which we extracted intact molar tooth organs, the poly(A)-containing fraction comprised 1-2% of the total RNA. The yield of poly(A)-containing RNA, and its ability to direct the synthesis of proteins greater than 25,000

TABLE 2

IDENTIFICATION OF "EXTRACELLULAR MATRIX COLLAGENS" WITHIN PRODUCTS SYNTHESIZED IN CELL-FREE SYSTEMS DIRECTED BY EXOGENOUS TOOTH MOLAR ORGAN mRNAs

Source of mRNA	Total ^{3}H-proline incorporation per 5 µg of mRNA (cpm) in 100 µl	Percent of Incorporated Isotope Collagenase-labile (%)
mRNA from tooth organ	23,600	60
globin mRNA	25,400	0
mRNA tooth mesenchyme	18,000	76
mRNA tooth epithelium	22,000	52
endogenous wheat germ system RNA activity	1,600	0

Messenger RNAs were isolated from either intact molar tooth organs or enzymatically dissociated tooth organs which resulted in epithelial and mesenchymal tissue isolates. In these studies we used 200 tissue isolates per experiment. This data is the mean value from three different experiments. Following translation *in vitro*, the reaction products were dialyzed exhaustively against triple distilled water prior to collagenase digestion (i.e. purified bacterial collagenase 1 mg/ml) (12). Incorporation due to endogenous wheat germ activity is routinely subtracted from total TCA-precipitable labeled protein.

daltons were found to be somewhat variable from one preparation to another. Under the experimental conditions employed in this study, ^{3}H-proline incorporation was linear at 30°C for at least 60 min and increased up to 180 min of incubation. Generally, stimulation of isotopic proline into polypeptides was directly proportional to the amount of RNA added up to 7-10 µg per reaction mixture (11).

Cell-free reaction products were analyzed on either 5% or 7.5% SDS-PAGE gels. These gels were compared to authentic extracellular matrix protein gels which contained either 6 M urea buffer extracts of non-labeled polypeptides or ^{3}H-proline labeled extracellular matrix proteins obtained from *in vitro* studies (7). Since most of the extracellular matrix proteins contain major post-translational modifications (e.g. glycosylation, phosphorylation, sulphation) (1), we anticipated that the tooth organ mRNAs would direct the

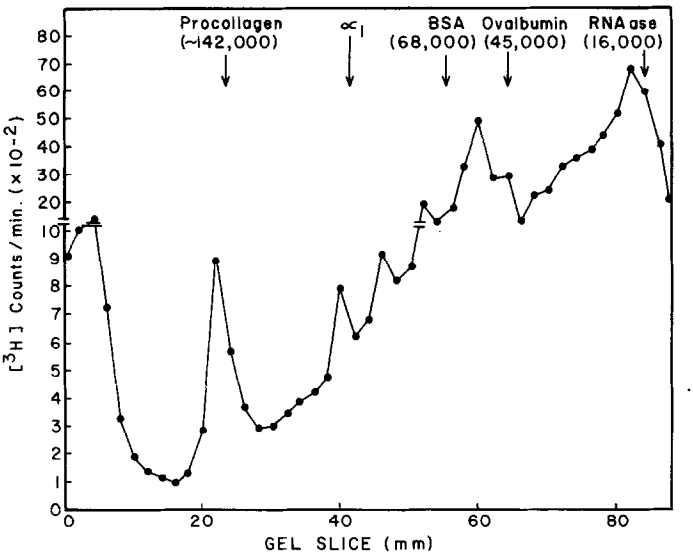

Figure 1. 5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (13) of the (^{3}H) proline-labeled protein synthesized in the wheat germ cell-free system in the presence (solid line, closed circles) of added embryonic molar tooth organ mRNAs. The standard proteins of known molecular weight electrophoresed in adjacent gels were purified type I procollagen alpha$_1$ chains (circa 142,000), collagen α_1 chains (~109,000), bovine serum albumin (BSA, 68,000), ovalbumin (45,000) and ribonuclease (RNAse, 16,000). The purified procollagen and α_1 chains were gifts from Dr. George Martin, National Institutes of Health SDS-PAGE has recently been used to identify four enamel proteins which characterize embryonic rabbit molar extracellular matrix: (1) 65,000, (2) 58,000, (3) 22,000 and (4) 20,000 molecular weight, respectively (7).

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synthesis of polypeptides in the cell-free system which would possess electrophoretic mobilities somewhat faster than the comparable proteins synthesized *in vivo*. Table 2 summarizes the collagenous characteristics of the *in vitro* translation products. Throughout these studies we suspected that the major

protein synthesized was procollagen, representing the synthesis of type I collagen by the odontoblasts. To test this assumption, we routinely determined the percentage of ^{3}H-proline incorporated which became dialysable following purified bacterial collagenase treatment. Approximately 60% of the translated products were collagenase-labile indicating that the tooth organ RNA extract contained procollagen mRNAs.

The poly(A)-containing RNAs derived from molar tooth organs directed the synthesis of six polypeptides with molecular weights up to approximately 145,000 daltons. The smallest polypeptide was 20,000. In two separate experiments the amount of ^{3}H-proline incorporated into the major 145,000 daltons protein comprised 50% of the total proteins synthesized *in vitro*. The *in vitro* products were compared to the proteins synthesized and secreted into the extracellular matrix *in vivo* (7). Each of the six polypeptides synthesized *in vitro* migrated in positions comparable, albeit somewhat smaller, to the major extracellular matrix proteins characteristic of dentine and enamel formation (Figure 1) (1, 2, 7).

The data presented here provide the first demonstration of the synthesis of tooth organ proteins in a cell-free protein synthesis system. We assume that the vast majority of mRNAs found within the epithelial and mesenchymal tissues were not detectable under these experimental conditions; the so-called "house-keeping proteins." We have shown that the translation products directed by poly(A)-containing RNAs have biological properties comparable to the extracellular matrix proteins associated with dentine and enamel formation. Current work is aimed at further purification of the enamel mRNAs as well as determining the ability of the unfractionated embryonic molar tooth organ messenger preparation to stimulate the synthesis of other extracellular matrix proteins such as dentine phosphoproteins and dentine collagen.

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