THE APPEARANCE OF STABLE POLYSOMES DURING THE DEVELOPMENT OF CHICK DOWN FEATHERS*

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The molecular sequence from gene to protein molecule (Brenner, et al, 1961; Gros, et al, 1961) presents at least two distinct alternatives for programming protein synthesis during development. Genes may be activated for extended periods to produce a continual supply of short-lived messenger-RNA (m-RNA) to translate for specific cell proteins (Scott and Bell, 1964). Alternatively, genes may function only during a brief period to program the protein synthesizing machinery with long-lived m-RNAs which act as persistent templates for the specific proteins of the differentiated cell (Bishop, et al, 1961; Scott and Bell, 1964).

Experimentally these two possibilities can be distinguished either by demonstrating that development is or is not stopped by inhibition of RNA synthesis (Gross, et al, 1963) or by demonstrating the presence or absence of stable m-RNA for the synthesis of cell specific proteins. The latter approach appeared possible in a tissue which passed through a developmental phase of rapid synthesis of one or a few cell specific proteins, (Bell, 1964). Embryonic chick down feathers begin to synthesize very large quantities of keratins during a relatively short developmental period between 13 and 14 days of incubation which appears to be a crucial time in feather development (Bell and Thathachari, 1963; Ben-Or and Bell, 1964; Bell and Humphreys, 1964). Since functional messenger is complexed with ribosomes to form polysomes (Warner, et al, 1963),

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any large fraction of m-RNA appearing at 13 days should be detectable in the developing feather as a new class of polysomes. Once this new species is detectable, the half life of its m-RNA can be measured after inhibition of RNA synthesis with Actinomycin D (AmD) (Penman, et al, 1963).

MATERIALS AND METHODS

Back skin or back feathers from embryos incubated at 37.6°C were collected in cold, sterile tyrode (see Bell and Thathachari, 1963 for a description of the skin and feathers). The tissue was treated with AmD* in vitro suspended in 20 volumes of 37°C Waymouth medium in a shaker flask. Polysomes were extracted from the chick tissue by a procedure used for HeLa cells (Penman, et al, 1963) except the chick tissue had to swell 15 to 60 minutes in the hypotonic buffer (RSB; 0.01 M Tris, pH 7.5, 0.01 M KCl, 0.0015 M Mg^{++}) and then be broken up by 5 to 10 quick strokes of the "loose" ball before complete disruption with the "tight" ball of the Dounce homogenizer. Sedimentation analyses were performed on 15 to 30% linear sucrose gradients in RSB centrifuged 180 minutes at 24,000 rpm at 8°C. Polysomes to be sonicated were collected from a gradient, pelleted at 38,000 rpm for 90 minutes in a Spinco SW 39 rotor, resuspended in RSB, and sonicated for one minute. After sonication they were layered on a second gradient and recentrifuged. Polysomal RNA was analyzed in SDS gradients (Gilbert, 1963).

Radioactive amino acids were incorporated into polysomes by a 4 minute incubation of the tissue in 37° Tyrodes solution with 2% dialyzed serum and 30 uc C^{14} algal protein hydrolysate (1 mc/mg. New England Nuclear Corporation) per ml. RNA synthesis was measured as TCA precipitable counts per O.D. 260 mu in phenol extracted, alcohol precipitated material (Sherrer, et al, 1962) from tissue incubated 2 hours in 37°C Waymouth medium with 5 uc C^{14} uridine (30 uc/mM) per ml.

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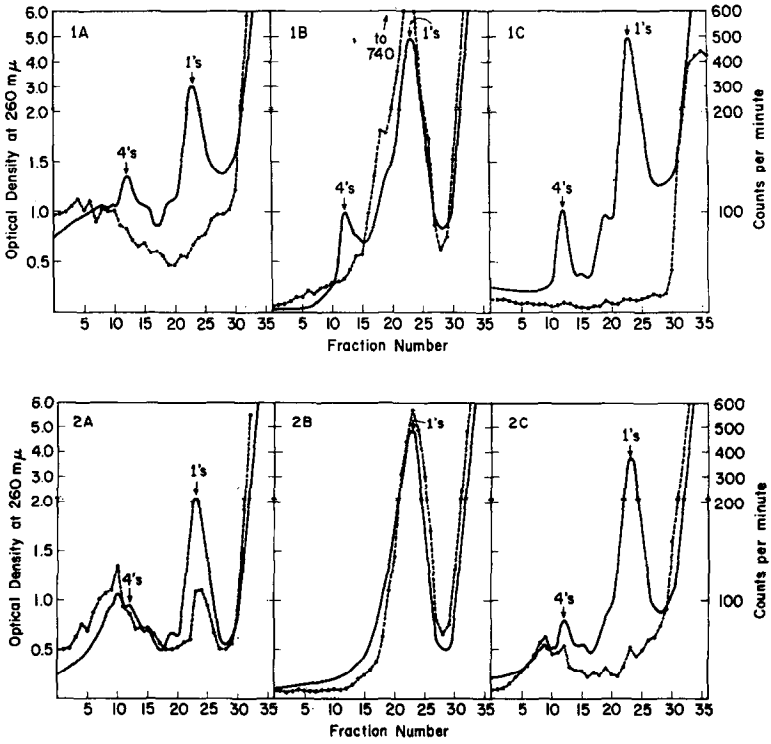
RESULTS

Cytoplasmic extracts from freshly isolated skin of five and one-half to 10 days and feathers of 11 to 15 days when sedimented in a sucrose gradient yielded curves of optical density at 260 mu characteristic of the ribosomal structures of many cell types, (Fig. 1a and 2a). Below the non-sedimenting material was a large 74S single ribosome peak and a series of lesser peaks with a long tail representing polysomes (polysome profile). In this tissue the polysome profile was almost constant throughout the developmental stages examined, changing only at 13 days. Before 13 days the 4 ribosome polysome peak strikingly predominated, after 13 days the five and six ribosome polysome peaks increased until by 15 days the 5's peak at least equaled the 4's



Figures 1 and 2 - Polysome profiles from zone sedimentation of radioactive amino acid labelled cytoplasmic extracts centrifuged 24, 000 rpm for 3 hours at 8°C on a 15 to 30% sucrose gradient. Solid line is optical density at 260 mu. Broken line is TCA precipitable counts per minute. Fig. 1-extracts from 13 day feathers. Fig. 2-extracts from 15 day feathers. A-freshly isolated tissue. B-extract A after treatment with RNase at 4°C for 30 minutes. C-tissue treated with Actinomycin D for 24 hours.

peak (compare Fig. 1a and 2a). Keratinization physically prevented extraction of polysomes from later stages. This relatively constant distribution of polysome sizes is characteristic of chick skin and feathers and does not occur in other chick tissue so far examined (Scott and Bell, 1964).

Radioactive amino acids are rapidly incorporated into the polysome regions of these gradients (Fig. 1a and 2a). Treatment of these extracts with ribonuclease quickly moved most of the O. D. and radioactivity into the region of the single ribosome peak. Most of the material recovered in the polysome region of these gradients are therefore clearly polysomes. However, in tissue 13 days and younger there was a distinct optical density peak, with no associated radioactivity, which resisted the ribonuclease and persisted in the area of the four ribosome polysomes (Fig. 1b). After 13 days ribonuclease moved all optical density and radioactivity from the polysome region into the single ribosome peak. (Fig. 2b).

TABLE I

Percent RNA synthesis in tissue cultured in vitro with Actinomycin D as compared with controls cultured an equal time without AmD.

time in culture	9 day skin with 30 ug/ml	11 day feathers with 30 ug/ml	13 day feathers with 60 ug/ml	15 day feathers with 60 ug/ml
2 hours	0.8	-	9	6
6 hours	0.4	-	3	2
24 hours	0.4	1	1	0.8

Upon 99% inhibition of RNA synthesis (Table I) by AmD in vitro, the polysome profiles of 9 day skin and 11, 13, and 15 day feathers began to decay rapidly. Polysome profiles of all tissues cultured without AmD remained essentially unchanged for more than 24 hours. After 12 hours in AmD the polysome profile from 9 day skin and 11 and 13 day feathers consisted of a sharp, prominent peak in the 4 ribosome polysome region on an otherwise low background (Fig. 1c). This peak persisted until the tissue began to disintegrate

at about 18 hours in 9 day skin and well beyond 24 hours in 11 and 13 day feathers. This persistent peak did not incorporate radioactive amino acids even when the tissue was incubated with label for 20 minutes (Fig. 1c). The ratio of optical density at 260 mu/280 mu at the persistent peak was 1. 9 which is the ratio of the single ribosome peak. Sonication of the persistent peak moved it to the single ribosome peak. The RNA of the persistent peak sedimented in a sucrose gradient exactly like the RNA of single ribosomes. This persistent peak therefore appears to represent a multiple ribosomal aggregate. The polysome profile of 15 day feathers decayed much as that of 13 day feathers except there were several peaks of larger long-lived polysomes along with the predominant peak in the 4 ribosome polysome region (Fig. 2c). Radioactive amino acids were rapidly incorporated into the long-lived 15 day polysomes. (Fig. 2c).

DISCUSSION

Maturation of the down feather is completed by the rapid deposition of large quantities of the structural proteins, keratins, between 13 and 17 days of incubation. These results suggest that before 13 days, during feather growth and morphogenesis, the feather cells are preparing for this final step by producing and storing 4 ribosomal aggregates as non-functional polysomes whose m-RNA is specifically protected against ribonuclease activity. When morphogenesis is almost completed at 13 days, this store of polysomes appears to be activated to produce the proteins necessary for maturation.

This interpretation raises several interesting questions. It assumes that the non-functioning 4-ribosomal aggregates which appear before 13 days are organized around m-RNA molecules and are the direct precursors of the stable, functioning 4, 5, and 6 ribosome polysomes occurring after 13 days. The appearance of 5 and 6 ribosome stable polysomes in addition to the original 4 ribosome polysomes may indicate random attachment and movement of ribosomes during translation of a single species of m-RNA or may indicate several species of m-RNA which had been previously complexed uniformly with 4 ribosomes in the non-functioning state. Several m-RNA species would be expected since more than one keratin protein is known for several tissues (Mercer, 1961), but

these possibilities have not been examined experimentally. Indeed, another plausible alternative is that the appearance of functional, stable polysomes at 13 days results completely or in part from the synthesis at that time of one or more new species of m-RNA.

Cytological identification of these non-functional ribosomal aggregates with the "clouds" of 200 to 250 A particles found in developing down feathers (Kischer, 1962) may be possible. These "clouds" were thought to be related to the keratohyline granules which build up in cells of mammalian keratinizing tissue before keratinization and then rapidly disappear as keratinization occurs (Brody, 1959). To our knowledge, nucleic acid in these granules has not been studied.

The interpretation presented demands three distinct controls which must operate in the regulation of the synthesis of these feather proteins. Genes must be activated early in feather development to produce m-RNA for the non-functioning aggregates. The preformed aggregate must be switched on, and the functioning polysome must be stabilized to function over the period necessary to complete deposition of sufficient protein.

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