Mechanisms of cell specialization

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In the preceding report, Dr. Massaro has reviewed lucidly a model for the transcription of the genetic code from DNA into RNA and the translation of the RNA message into protein on the complex of ribosome-transfer RNA (tRNA)-messenger RNA (mRNA). This model provides a general basis for the redefinition of developmental cell specialization in terms of protein synthesis. In adopting these concepts, cell specialization can be conceived of as the result of the synthesis of cell-specific proteins such as hemoglobin, myosin, collagen, or the lens proteins; of the synthesis of cell-specific products, such as gangliosides or hormones, by cell-specific protein enzymes; and of the synthesis of common macromolecules, such as mucopolysaccharides or lipids, in specific quantitative proportions. Increasing evidence suggests that, as the assortment of macromolecules in a cell is being synthesized, assembly into larger aggregates of subcellular particles and organelles can occur by virtue of the characteristic properties of the macromolecules themselves. Examples are the assembly of ribosomes from their molecular components, of muscle filaments, or collagen fibers, or of cell membrane structures. In this sense, protein synthesis is here regarded as the first step in the structural and functional organization of macromolecules into subcellular units and, eventually, in cellular and supracellular organization as well.

Another prefatory and more general consideration should be added here. In developing tissues, the full expression of cell specialization is usually preceded by a period of rapid proliferation. In some instances, no cell-specific proteins are formed during this period of active cell replication. For example, no myosin is synthesized in the myogenic cells of skeletal muscle as long as the cells proliferate, but myosin appears after cell division ceases and fusion into multinucleate fibrils takes place. However, in developing heart cells, some myosin synthesis apparently takes place even during cell proliferation, and at least a limited production of cell-specific proteins has been claimed to occur in other cell types as well. Although cell-specific proteins may be synthesized during the proliferative period of some cells, the production of cell proteins required for cell maintenance and cell replication must be the dominant form of synthesis during this period of development. In contrast, synthesis of cell-specific proteins becomes in general more prominent after cessation of proliferation.

Having added to Dr. Massaro's paper these footnotes on general aspects of cell differentiation, I would like to suggest that the biologist's interest is very much concerned with both the universal and the particular. The biologist asks how, in the course of biological evolution, the same basic molecular mechanisms, e.g., the ge-
netic code or the energy-rich phosphate bond, were used again and again to maintain as different forms of life as a single-cell bacterium or a Shakespeare, or within the same organism tissues as different as muscle, skin, lens, or retina. Because of this dual interest in the study of life, it is proposed to turn now from the discussion of general principles of cell differentiation to the question of what we know about the applicability of these principles to the analysis of the development, specifically, of the tissues of the eye. I would like to approach this assignment by asking what answers we can give today to two questions: (1) How far have we come in the redefinition in molecular terms of the development of the tissues of the eye? (2) What can be said about factors which control the transcriptional mechanisms during eye-tissue development?

Molecular basis of the development of the tissues of the eye

Three examples may illustrate different forms of cell-specific protein synthesis as they occur in the tissues of the developing eye. In one form of differentiation, the increase of the cell-specific protein occurs at a rapid rate but during a short period of development. Hence, the final protein level which is attained during such a short time span remains relatively low and still within the range of the non-cell-specific proteins. This form of protein formation is found in such tissues as the liver, which produce a great number of cell-specific enzyme proteins for the diverse metabolic functions of this tissue. During development of the tissues of the eye, the dramatic appearance of the enzyme glutamine synthetase in the chick retina seems to fall into this category of developmental protein formation. In this tissue, no activity of this enzyme can be detected until 10 days of incubation, after which activity increases slightly up to the fifteenth day. From the sixteenth day to one week after hatching, the specific activity of the enzyme increases 200-fold and remains at about this attained level of activity during the remaining life-span of the chick. The abrupt increase in glutamine synthetase activity occurs also in tissue cultures of embryonic chick retina. There are reasons, discussed later on, to believe that the increase in enzymatic activity of glutamine synthetase is due to de novo protein synthesis. Since the enzymatic assay permits detection of very small quantities of protein, the final level of glutamine synthetase activity corresponds, probably, to a small amount of protein of the order of magnitude of several other enzymes in the retina involved in intermediary metabolism. Therefore, one can tentatively suggest that the appearance of glutamine synthetase represents a type of development of cell-specific proteins which may involve a relatively small part of the total protein-forming system (mRNA, tRNA, and ribosomes) of the developing retina.

In a second type of differentiating cells, the concentration of a few cell-specific proteins increases to levels considerably in excess of the concentrations of any single non-cell-specific protein. The accumulation of hemoglobin is an outstanding example of this. Also, the major portion of the body mass of vertebrate animals is formed by a prolonged increase in cell-specific proteins like myosin, actin, and tropomyosin in muscle, and collagen in the connective tissue of skin and bones.

An attempt to define in quantitative terms this form of differentiation in one of the eye tissues has led us to the study of protein formation in the cornea and sclera of the developing chick embryo. This tissue proved useful in following the changes which occur during the terminal phase of differentiation—which includes cessation of cell proliferation and initiation, acceleration, and decline of synthesis of collagen, which is regarded here as the main cell-specific protein of the corneal stroma cells.

The development of the corneal stroma was investigated in detail. In the chick
embryo, the cells of the corneal stroma begin to migrate from the head mesenchyme into the corneal area on the sixth day of development. From measurements of DNA in the stroma, one may conclude that the cell number in the stroma increases until about 13 to 14 days of development, reaching a maximal level of 5 to 6 μg of DNA per stroma which is maintained during the remaining phase of development. To what extent this increase in cell number is due to a continued cell migration or to proliferation of cells which have already migrated to the stroma has not been investigated as yet.

The relationship between the cell number, the amounts of protein, and incorporation of amino acid into noncollagen protein and collagen in the developing corneal stroma can be seen in the graphs of Fig. 1. The level of noncollagen protein per unit of DNA increases from 2.1 μg on the ninth day to 4.6 μg on the twenty-fifth day of development. The corresponding figures for collagen are 0.8 and 12.1 μg, respectively. The noncollagen proteins increase at a slow and constant rate, whereas collagen accumulates at an increasing rate up to the sixteenth day and at a decreasing rate from the sixteenth day to the twenty-fifth day of development. From 9 to 12 days of development, the content of noncollagen protein in the stroma is higher than that of collagen. The amount of the two classes of proteins becomes about equal on the twelfth to thirteenth day. At 20 days of development, collagen reaches a level which is about three times higher than the corresponding value for noncollagen proteins.

These analytical determinations of amounts of noncollagen and collagen proteins in the corneal stroma were compared with measurements of the uptake of radioactively labeled amino acids into the two protein fractions. The noncollagen fraction showed a low rate of incorporation which did not change significantly during development. Some loss of label from the noncollagen fraction one day after injection of label into the intact embryo indicated a measurable turnover of the proteins in this fraction. In contrast, the incorporation rate in collagen reached a very distinctive peak at 16 days of development and the absence of a loss of label indicated a minimal turnover of collagen. The peak for the rates of amino acid incorporation into collagen coincides with the steepest part of the collagen-accumulation curve. After the sixteenth day, both the rate of incorporation and the slope for accumulation decline in a comparable fashion. One can suggest that, in the case of corneal collagen, accumulation and synthesis are equivalent parameters. A similar course for collagen accumulation has been observed for the sclera, but no incorporation studies have been carried out as yet in this stepchild of biological and ophthalmological research.

![Fig. 1. Comparison of changes in DNA, collagen, and noncollagen protein contents and in amino acid incorporation in collagen and noncollagen protein in the stroma of the developing chick. Top, rates of incorporation per DNA of glycine 14C into collagen and noncollagen protein. Center, amounts per DNA of collagen and noncollagen protein nitrogen. Bottom, amounts of DNA per stroma.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933618/)
Cornea and sclera are tissues in which the absolute quantities of cell-specific proteins (collagen) which are produced during development are considerably in excess over the total amount of the nonspecific proteins. This suggests that in this case a substantially greater portion of the protein-forming systems (mRNA, tRNA, ribosome) becomes committed to elaboration of cell-specific protein than, for example, in the retina.

An exceptionally high increase of cell-specific proteins (α, β, γ crystallins) is found in the developing lens. Of all tissues, the fully differentiated lens is known to have the highest protein content (about 30 per cent), with the lens crystallins accounting for most of this protein level. The exact relation of accumulation and amino-acid incorporation has not been worked out as yet for the crystallins. However, even the measurements of contents of lens protein which are available at the present time show that the lens is perhaps one of the most extreme cases of cell differentiation in terms of accumulation of cell-specific proteins. This holds in respect to both the excess of crystallins over the nonspecific proteins and the long duration of the developmental period during which accumulation of crystallins continues. In following the interpretation of the differentiation of retina and cornea, one would assume that in the lens a very large part of the protein-forming system may be committed to the synthesis of the crystallins.

By emphasizing the differences in the course of protein synthesis in the retina, cornea, and lens, it was intended to show that such a reinterpretation of cell development is quite feasible for the tissue of the eye. As the next step in such a study one is led, obviously, to the question about the factors which control protein synthesis during development of the eye tissues and bring about the differences in the patterns of differentiation which have been discussed so far. In the following section it will be attempted to show that guidelines for the investigation of controls of protein formation in developing eye tissues have been established and that, in certain respects, eye tissues may offer considerable advantages for such analyses.

**Control of transcription and translation**

As a preface to this section, a brief detour into classical experimental embryology may be appropriate. It should be recalled that lens formation has been of great importance in the study of embryonic induction. The transformation of the primitive ectoderm into lens epithelium under the influence of the eyecup, the primordium of the retina, has been given almost as much attention by students of embryonic induction as Speman's famous organizer system. In terms of modern molecular biology, lens induction must mean that during their juxtaposition the eyecup tissue exerts an influence on the primitive ectoderm which ultimately activates transcription and translation for lens proteins and prevents formation of non-lens-cell protein, e.g., hemoglobin, collagen, keratin.

The search for the factors which exert such profound effects on transcription and translation during these early stages of development has been, and still is, perhaps the most challenging but also the most frustrating problem of developmental biology. Although protein fractions have been found which seem to have effects similar to Speman's organizer, it is often difficult to evaluate whether these are specific or unspecific effects. Also, the absence of quantitative criteria for the response of the induced tissue hampers further analysis. Lens induction may be a favorable system for a study of induction for several reasons. Compared to chorda-mesoderm, which induces neural structures, the lens-inducing tissue, the eyecup, is further along in its development and it may yield fewer and more distinctive macromolecular fractions with inducing properties. The inducing material from the eyecup must be transported to the reacting ectoderm since the retinal primordium and the reacting ectoderm are separated by a small but distinct inter-
cellular space. It may be easier to attempt separation and characterization of such cell-separated inducing material than it would be to interpret direct cell-surface interaction. Finally, the transformation of ectoderm into lens cells can be followed histologically and the first appearance of crystallins can be detected and quantitatively measured, e.g., by immunological methods. Hence, it may be more rewarding to set about solving the knotty induction problem in the lens system rather than in neural induction.

While the problem of this early control is still in the realm of dreams, work with the lens has recently opened very concrete insights into regulation of protein synthesis during development. In microbial systems, mRNA which transmits the code instructions from the DNA to the ribosomal site of protein synthesis is very labile. mRNA is destroyed in a matter of minutes and extended protein synthesis requires continued synthesis of the respective messenger molecule. Any inhibition of messenger synthesis leads to a decline in protein formation. If protein synthesis during development of animal cells depends on the presence of such labile messenger molecules, impairment of RNA synthesis must lead to cessation of protein formation. In this case, control of the mRNA synthesis, that is of transcription, would be the main point of developmental regulation. The discovery that actinomycin D in low concentrations inhibits the transcription of the DNA code into RNA has provided a tool with which to investigate the dependence of certain steps of development on RNA synthesis. In the developing eye tissues, the effect of the antibiotic was investigated in the lens and in the retina. Investigating lens development, Papaconstantinou and his associates showed that formation of lens proteins in the epithelial cells is strongly inhibited by actinomycin D, whereas synthesis of these proteins in the lens fiber cells is actually stimulated by this antibiotic. The observations by Papaconstantinou and similar results obtained by Scott and Bell led to the conclusion that formation of lens proteins before fiber formation depends on labile mRNA and requires rapid mRNA synthesis. In contrast, formation of the proteins in the lens fiber is maintained by a stabilized form of mRNA. Such a stabilization of mRNA has been observed in several developing animal cells and is apparently characteristic of protein synthesis during late phases of development in higher forms of life. The work with the lens provides a very clear and detailed account of the transition during development from protein synthesis with labile RNA to synthesis with stable RNA. Therefore, the lens may well become a favorite tissue for the study of the nature of messenger RNA stabilization, for experiments performed in

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**Fig. 2.** Schematic representation of the relationship between cellular and molecular changes during transformation of lens epithelium into lens fibers. (Redrawn from Papaconstantinou, J.: Science 156: 338, 1967.)
an effort to labilize a stable mRNA or to prevent the stabilization of a still-labile mRNA molecule.

The research on lens development discussed so far demonstrates that morphological and cytological events are well correlated with protein synthesis and with changes in the transcription and translation of genetic information which control these syntheses during the development of the lens tissue (Fig. 2).

Experimental embryologists have found still another form of lens development which may open rather unusual possibilities for the study of the molecular basis of cell specialization. I am referring here to the interesting phenomenon of regeneration of the lens which was described by Wolff in 1894 (Wolffian regeneration). It has been intensively investigated by Stone and by Reyer and in recent years also by Yamada and his associates. This remarkable process occurs in the eye of certain amphibians. Upon experimental removal of the lens a new lens is formed from the iris cells which are located in the dorsal segment of this tissue. This observation is important because it may demonstrate the only case of transformation of one fully differentiated tissue into a quite different fully differentiated tissue. This process has been recently called transdifferentiation. In following the main phases in this transformation, it is observed that on the second day after removal of the lens an increased synthesis of RNA and protein occurs in the cells of the dorsal rim of the iris. On the fifth day, incorporation of thymidine into the nuclei suggests initiation of DNA replication which coincides with the observation of mitoses, signaling the beginning of a proliferative phase of the iris cells. During the subsequent five days, proliferation continues and the pigment is lost from the cells. Toward the end of this period, the inner cell layer assumes the shape of lens fiber cells. These cells no longer show proliferative activity and appearance of lens proteins can be demonstrated with immunological techniques. The outer layer of this transforming tissue retains proliferative activity and becomes the source of secondary lens fibers similar to the events in normal lens development. When actinomycin D is administered, the lens fiber cells continue forming proteins while the cells of the anterior lens epithelium degenerate.

This sequence of events in Wolffian regeneration can be interpreted in terms of mRNA stabilization as in normal lens development; the early development of lens cells, being dependent on labile, rapidly synthesized RNA and protein formation, becomes independent of rapid mRNA synthesis in the lens fiber cells because of mRNA stabilization. However, the main point is that a qualitative change in the transcriptional process has been achieved. Transcription for synthesis of iris proteins has been "turned off" and transcription for synthesis of lens proteins has been "turned on"; the "iris DNA code" has been blocked and the "lens code" has been activated. This is analogous to the qualitative change in the transcribed portion of the DNA code which occurs in embryonic induction.

In the earlier discussion of embryonic induction, reference was made to the difficulties encountered in the isolation of the tissue components which direct changes in transcription. Therefore, experiments should be mentioned which show that tissue factors control the transformation of the iris into lens during Wolffian regeneration. When iris was explanted in tissue culture, no transformation was observed; but, when the dorsal rim of the iris was implanted into the anterior eye chamber after lens removal, extensive lens fiber formation did occur. Further experimentation pointed to the retina as the source of the active material in the aqueous humor of the anterior chamber. In the presence of a lens no Wolffian regeneration was observed. It has remained unknown whether under these conditions the retina factor is not formed in, or not released from, the retina, or whether retina and lens factors combine and become an inactive complex.
Several intriguing possibilities for further research appear. If the retina material is transported in the aqueous humor by free diffusion, it may be prepared directly from this medium. It may be extractable in more substantial quantities from the retina itself and its yield may increase after removal of the lens. Finally, specificity of the factor could be ascertained by the antagonism of retina and lens factors. One could think even of maintaining iris, retina, and lens in different combinations in tissue culture in order to study their interactions in these simplified systems.

In the outline given so far, the discussion of endogenous factors which activate or inactivate main steps in the transcription-translation mechanism during the development of the tissues of the eye have remained quite hypothetical. It is as if one were sitting in a car, knowing that it can be driven away at variable speed and that the "transcription" and "translation" of the latent energy of combustion fuels makes the engine turn. What is lacking for the purposeful operation of the car is the understanding of the principle of the electric ignition and the key needed to turn on the starter. That the quest for key substances which start transcriptional and translational processes in the developing cell can lead to concrete results is indicated in two following examples.

It was pointed out earlier that during differentiation of the retina the enzyme glutamine synthetase is produced at a rapid rate. In cultures of retina the rapid synthesis occurs only if serum from adult animals is present in the culture medium; fetal serum has no such effect. In searching for the active component of adult serum, several hormones were tested. Among these, only cortisone was found to increase glutamine synthetase activity to the same extent as adult serum, and it was assumed that this steroid is the active component of adult serum. The response to adult serum was found to be inhibited by actinomycin D. Therefore, it can be assumed that serum and possibly cortisone activate transcription with increased production of messenger RNA for glutamine synthetase activity.

The preceding example should not suggest, however, that the common hormones (such as cortisone), which have a broad range of effects, are necessarily the main agents for transcriptional or translational control during development. Recent work has uncovered proteins which seem to play an important role as specific developmental regulators. One of these substances enhances the growth of spinal and sympathetic ganglia by stimulating apparently actinomycin-inhibited transcriptional processes. Whether these or related agents may exert an effect on the development of the retina as part of the central nervous system remains to be investigated. Another protein hormone has been isolated which stimulates the separation of the eyelids in the newborn rat. Analysis of this effect showed that this epidermal differentiation factor, which also stimulates eruption of the teeth, promotes keratinization—an essential step in lid separation. Since the effects of this factor are insensitive to actinomycin D, transcriptional control seems unlikely, and a more effective use of mRNA on the translational level may lead to the observed increase in synthesis of epidermal proteins.

This survey was opened with the question whether the general model for the expression of gene action can give guidelines for the analysis, specifically, of the development of the tissues of the eye. The examples given in this outline should indicate that, during the few years since molecular mechanisms of genetic transcription and translation were originally postulated, the applicability of this type of analysis to the investigation of the development of the tissues of the eye has become quite feasible and profitable. Indeed, it may very well be that some of the eye tissues, e.g., the lens, may prove to be exceptionally promising systems for further studies of the molecular control of normal and abnormal embryonic development.
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**Regulation of ocular morphogenesis**

**Alfred J. Coulombre**

During development of the vertebrate eye, a large number of tissues assemble in time and space to form an organ with a geometry which is in accord with the tolerances required by the law of optics. For over half a century, experimental embryologists have identified one after another of the factors which control the size, shape, position, orientation, and internal organization of developing ocular tissues. It is now abundantly clear that the remarkably precise architecture of the eye is achieved by an orderly, if complex, chain of influence among its tissues during the embryonic stage. Each tissue may be studied as a potential source of influence on some of its surrounding tissues. Each tissue may, in turn, be studied as a target of influences emanating from some of the surrounding tissues. These interactions among ocular tissues occur, in most vertebrates, in the dark, therefore removing the possibility that light input to the eye might play a role in regulating morphogenesis.

Enough information has now accumulated to make possible the construction of tentative flow sheets which describe in fairly precise terms the sequence of interactions by which information which is ultimately encoded at the genetic level achieves phenotypic expression. Such flow