Organogenesis

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Preface

"Organic form—the architecture and texture of organisms—is better known than understood."
Weiss, 1960

Those charged with arranging the program of the International Conference on Organogenesis believed that the time had come for re-emphasizing the study of development "beyond the ribosome." Acting under the conviction that ample attention was being given to problems of coding and protein biosynthesis, Professor Etienne Wolff, president of the International Institute of Embryology under whose auspices the conference was held, invited the organizing committee to develop a program that would center attention on supermolecular aggregates, cells, and cell associations. Thus at the conference the primary focus was the behavior and interactions of cells and cell groups in the fashioning of complex, ordered tissues.

Within the International Union of Biological Sciences, the International Institute of Embryology represents developmental biology. In addition to its publishing and research programs, centered in the Hubrecht Laboratory, the institute has convened a series of successful international conferences. Happily, the International Conference on Organogenesis did not prove to be an exception. Its success was attested by the genuine enthusiasm of the four hundred participants and the prompt, willing cooperation of speakers, discussants, and other contributors to this volume.

The conference was held in Baltimore, Maryland, September 6–12, 1964. The International Institute of Embryology and the organizing committee are deeply indebted to the hospitality of the Carnegie Institution of Washington, The Johns Hopkins University, and the local committee, for an attractive setting; and to the National Science Foundation, the International Union of Biological Sciences, and Holt, Rinehart and Winston, Inc., for grants making the meeting possible.

JAMES D. EBERT
Chairman, Organizing Committee

Baltimore
February, 1965

Editors' Introduction

"How all's to one thing wrought."
Gerard Manley Hopkins "On a piece of music"

Our purpose as developmental biologists is to understand the causal mechanisms underlying the processes of growth, differentiation, and morphogenesis. It is now accepted as a truism that events occurring at the level of the molecule, the cell, and the cell group or tissue operate simultaneously and coordinately to influence these processes. Thus, we cannot expect to derive satisfying explanations of how an eye or a limb or a heart develops by learning about the inductive interactions of the component tissue layers alone, or by knowing only of the microscopic behavior of the cells involved, or by learning what enzymes are active or what nucleotide sequences are transcribed. Only when we have access to information at all these levels—and can synthesize it—can we truly understand how an organ or an embryo develops.

Fortunately, there is a tendency for information at different levels to interact. When, through advances in instrumentation or techniques, a new approach is introduced, it is at first directed by existing information. What the ultrastructural or molecular biologist initially looked for—the problems he attacked, the questions he asked—was in large part guided by his knowledge of the properties of cells and cell groups previously determined with less refined techniques. However, as we now gain greater insights into the mechanisms of synthesis, interaction, and structural characteristics of macromolecules, these ideas will in turn increasingly direct the questions that are asked at the cellular and supracellular level.

It is our hope that the present volume will act as a catalyst in this interaction of information, leading toward the synthesis of ideas, derived from various levels of investigation, into a more comprehensive understanding of the problems of the development of organs and tissues.

As editors, we have attempted in *Organogenesis* to recognize the needs of three major groups of readers: investigators in developmental biology, advanced students and teachers of embryology, and biologists in other fields. Investigators require definitive review articles covering their own and related areas to aid them in digesting and creatively synthesizing the deluge of litera-

ture with which they are inundated today. The other readers also desire critical and comprehensive reviews, but may, in addition, need sufficient introductory material to make these reviews meaningful. We have striven to have the chapters in this book fulfill both of these requirements wherever possible.

The contributing authors of this volume must receive ultimate credit for whatever success it achieves. To them we express our thanks for their promptness in submitting manuscripts, for their willingness to accept editorial suggestions, and above all, for their remarkable expertise in their respective subjects.

ROBERT L. DEHAAN
HEINRICH URSPRUNG
Editors

Baltimore
April, 1965

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SECTION I Basic Mechanisms

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Genes and Development

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When biologists first began to wonder about the role of genes in development, the presence of a gene responsible for a trait could be inferred only if a mutation occurred that led to alteration of the trait. Fortunately for the science of developmental genetics, a fair number of such mutations had been described by breeders, and their mode of inheritance analyzed by geneticists through pedigree studies. In those early days it became evident that the central task of developmental geneticists was to be the analysis of the mode of action of genes, mutant and normal.

At first, gene action was assessed at very different levels of biological expression. The phene "waltzing" of the house mouse (Guaita, 45), for example, is a behavioral one, and the responsible gene (*v*) expressed itself on a behavioral level. Another factor, Danforth's short tail (*Sd*) on the other hand, was found to act early in embryonic development on tissue interactions. A refined study revealed the fact that in this mutant the ureters fail to grow, inductive interactions do not occur, and as a consequence, kidneys do not develop (Gluecksohn-Schoenheimer, 39). Yet another gene of the mouse, dwarf (*dw*) expressed itself on a histological-endocrinological level. The eosinophilic cells of the anterior pituitary of these mice are histologically abnormal and apparently fail to produce thyrotrophic and adrenocorticotrophic hormones. The consequences are dwarfism and sterility (Smith and Macdowell, 93). A fourth category of gene action was measured on the chemical level. A large number of mutants which affect anthocyans and their derivatives in petals (Alston and Hagen, 4) were found in flowering plants. The well-known mutant genes controlling ommochromes and pteridines in the eyes of *Drosophila* also belong in this category (Ziegler, 116).

These studies clearly indicated that gene action can be observed on many different levels of biological organization. But geneticists early postulated that a primary gene action underlay the different phenotypic expressions

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of a mutation. According to this concept of unity of gene action (Grüneberg, 43), it appeared sufficient to assume that a gene produced one effect, which automatically, through the channels of development, affected subsequent steps and ultimate traits. This concept explained in a very simple fashion the frequent phenomenon of pleiotropic gene action, because it was now easy to picture an entire syndrome of effects as the result of a single shift from normal to mutant on the gene level. Developmental geneticists undertook to build pedigrees of causes, starting at the level of the complicated syndrome, and tracing the elements back in development to the unifying and simple initial genetic defect. However, the existence of this genetic defect was still a prerequisite for such a study because the existence of a normal allele could be inferred only when the consequences of an abnormal allele became manifest.

Soon the concept of unity of gene action was carried an important step further. Not only was it assumed that a given gene brought about its manyfold consequences by a primary action, it was postulated now that the primary action of all genes was of one kind only. This important concept was primarily a result of biochemical studies on mutants of *Drosophila* and *Neurospora* and was highlighted by the well-known one-gene–one-enzyme hypothesis (Beadle, 10). Several new slogans followed the one-gene–one-enzyme hypothesis, until now, after two decades of intensive research, it has metamorphosed to the one-operon –one-messenger theory (Martin, 75).

The present theory of gene action is a result of brilliant genetic research, primarily on microorganisms. It is therefore not surprising that the first speaker of the 1964 International Conference on Organogenesis was not an embryologist, but a microbial geneticist, Francois Jacob of the Institut Pasteur in Paris. Jacob, with his colleague Jacques Monod, is considered the father of the current dogma of gene action, which will be described in the following section.

But before turning to this discussion, we should recapitulate the predogma state of developmental genetics. It has been shown, beyond doubt, that cellular differentiation and development of higher organisms is under genetic control. It was found that the action of genes affecting development is pleiotropic. In the construction of pedigrees of causes, various levels had been reached; a few analyses were successful in tracing pleiotropic systems down to the level of an enzyme deficiency.

This introduction is brief and incomplete and therefore does injustice to many investigators whose thoughts and experiments in the area of cytology, histochemistry, cytogenetics, biochemistry, and other branches of biology helped construct the current view of the sequence leading from DNA via RNA to protein (Caspersson, 29; Brachet, 21; and many others). Their work has been described in a number of monographs and texts and cannot be reviewed here. These books should not be put on the shelf however, for soon developmental biologists will want to use monographs such as those by Grüneberg (43, 44), Hadorn (47), and Waddington (109, 110) when it will be important again to build predigrees of causes – leading from genes to phenes, this time.

The Dogma of Gene Action

The concept of gene action, as presented by Jacob and Monod (59) in 1961, consists of two major parts. First, it states that the genetic information of an organism, encoded in the nucleotide sequence of DNA, is transcribed into a complementary nucleotide sequence called messenger-RNA (m-RNA). This m-RNA then establishes a temporary union with ribosomes, and the nucleotide sequence is translated into the amino acid sequence of a specific polypeptide. Second, the dogma states that the synthesis of m-RNA is regulated by specific repressors which are products of regulator genes. The repressors are thought to act by becoming engaged with the operator site of the gene, their affinity to the operator being determined by the concentration of metabolites, so-called effectors. An operator is the control site of a cluster of structural genes. When the operator is open, all genes of the operon synthesize m-RNA; when it is closed, none do.

We shall describe some of the evidence advanced in favor of the dogma, and point out some of the difficulties that await solution. This discussion will deal with microbes, for the most part. A special section discusses the dogma's applicability to higher organisms.

Transcription

When a culture of *Escherichia coli* is infected with T_2 bacteriophage in the presence of P^{32} orthophosphate, within a few minutes label can be recovered that has become incorporated into RNA (Volkin and Astrachan, 108). The ratio A + U/C + G of this RNA is about 1.7—that is, almost identical to the base-ratio of T_2 DNA (1.8), but quite different from that of *E. coli* DNA (1.0). From this observation it appeared that the phage DNA primed the rapid synthesis of a specific, complementary RNA. This notion has since been substantiated by a number of elegant experiments. Hall and Spiegelman (49) prepared P^{32}-labeled m-RNA from T_2-infected *E. coli* and mixed this preparation with H^3-labeled T_2 DNA, the two strands of which had previously been separated from one another by heat. The mixture was cooled and centrifuged through a cesium chloride density gradient. Three bands formed and the analysis showed that one of them was DNA, the second, RNA, and the third, a molecular hybrid containing both H^3 and P^{32} labels and having a density intermediate between DNA and RNA. When, as a control, bacterial DNA was used as the partner of the m-RNA in the mixing experiment, no such hybrid DNA-RNA molecules were detected. It is thus apparent that the T_2 DNA primed the synthesis of a specific m-RNA which by virtue of its complementary base sequence hybridizes with the DNA. Such a synthesis is also possible in vitro, using bacterial RNA polymerase in the presence of ribonucleotide triphosphates (Weiss and Nakamoto, 111; Hurwitz et al., 57). Depending on the type of DNA primer utilized in such an experiment, the base sequence of the newly synthesized RNA varied. This base sequence can be determined only indirectly at the present time by a procedure called nearest neighbor sequence analysis (see Kornberg, 62, for review), which does not permit determination of the complete nucleotide sequence. However it was found, for example, that the position of cytidy-

late in the new RNA was specifically determined by the DNA that was used as primer, which strongly indicates that the exact base sequence of the m-RNA is primed by DNA. Methods are now becoming available that, hopefully, will permit a direct visualization of nucleotide sequence by electron-microscopy (Beer, 12; Moudrianakis and Beer, 81).

With these facts apparent, it became important to find out in more detail how transcription operates. DNA is a double helix of two strands with complementary base sequences. If the base sequence of m-RNA determines the specificity of the message, then two quite different messages could be read, one from each DNA strand. A number of experiments have shown, however, that the genetic information is read from only one of the two strands of the DNA double helix (Champe and Benzer, 30; Bautz and Hall, 9). Using the RNA-DNA hybridizing technique, it was possible (9) to prepare phage DNA-specific m-RNA. A quantitative analysis of nucleotides present in this RNA was then done. If both DNA strands had been read during transcription, and if they both produced an equally stable copy, then the RNA should have contained equal amounts of, for example, guanine and cytosine. This was not the case, however. There was more guanine present than cytosine, which indicated that only one strand of the DNA had been read. This notion has since been confirmed by a variety of experimental procedures (Hayashi et al., 51).

Translation

Pardee (88) had shown that protein synthesis comes to a halt when RNA synthesis is stopped. Through the use of radioactive amino acids it was then found that amino acids become first incorporated into protein associated with ribosomes (Littlefield et al., 68). Nomura and co-workers (87) showed that newly formed m-RNA is associated with ribosomes. From these observations the idea arose that ribosomes function as assembly plants, where m-RNA encounters amino acids, precursor molecules for polypeptide synthesis, and where new proteins are synthesized. The question then arose as to whether ribosomes carry any information themselves, or whether they are unspecific workbenches. This query was answered in an experiment by Brenner and co-workers (23). These authors prepared "heavy" ribosomes by growing *E. coli* in a medium containing N^{15} and C^{13}. These heavy ribosomes can be recognized and separated from "light" ribosomes by centrifugation. Bacteria containing heavy ribosomes were then infected with T_2 phage and immediately transferred to a light medium containing radioactive nucleic acid and protein precursors. When the ribosomal populations of such cultures were examined, it was found that no light ribosomes had formed. Furthermore it was seen that the newly formed m-RNA and protein were associated with heavy ribosomes. These had previously been involved in the synthesis of bacterial proteins, and were now used to fabricate phage protein under the direction of the phage m-RNA. Thus the specificity for protein synthesis resides in the m-RNA, not in the ribosomes.

This does not imply that their RNA sequence is unspecific. In fact it has been shown by the molecular hybridization technique (Yankofsky and Spiegelman, 114) that ribosomal RNA

is complementary to the nucleotide sequence of two cistrons in the bacterial genome.

The actual translation mechanism has been studied in more detail by Hoagland and co-workers (53) in rat liver, and by Berg and Ofengand (15) in bacteria. When amino acids are added to a high spin supernatant containing ATP and soluble-RNA (s-RNA), complexes form between s-RNA and amino acids, under the guidance of amino acid-specific activating enzymes. The complex is then transferred to ribosomes, a step that depends on the presence of GTP. Because of this function in protein synthesis, s-RNA is also called transfer-RNA (t-RNA). There is at least one t-RNA species for each amino acid. The t-RNA–amino acid complexes become attached to m-RNA on the ribosomes, peptide bonding occurs, and t-RNA is released. Apparently m-RNA recognizes the t-RNA moiety of the complex, not the amino acid. This was proved by an ingenious experiment (Chapeville et al., 31). First, cysteine was complexed to its t-RNA. The cysteine was then desulfhydrylated to alanine by Raney nickel. Thus an unusual complex was produced, namely, a t-RNA carrying the wrong amino acid. This complex was introduced into a protein synthesizing system containing a synthetic messenger, polyuridylicguanylic acid (poly-UG), which from other work is known to direct the incorporation into protein of cysteine, but not of alanine. Would this messenger now recognize the cysteine-specific t-RNA of the complex and incorporate alanine? Or would it recognize alanine, and therefore not incorporate it? The first alternative was observed, thereby proving that the recognition occurs between messenger and the transfer-RNA moiety of the complex.

The next, very important question to be solved was a language problem. Which are the words used in the process of translation? In molecular terms, which nucleotide sequence of the message is translated into which amino acid in the protein?

Artificial messengers have been instrumental in solving this important question of the code. The letters of m-RNA are known; they are A, U, G, and C. In order to specify 20 words, the letters must be written in groups of at least three, a code that would actually allow 64 words. But the next shorter code—that is, two letters per word, would specify only 16 words. A frontal attack on the problem was initiated by Nirenberg and Matthaei (85) and by Lengyel, Speyer, and Ochoa (66). A bacterial homogenate containing ribosomes, t-RNA, an energy source, and enzymes can incorporate radioactive amino acids into polypeptides in the presence of synthetic polynucleotides. When poly-U was used as the messenger in this system, phenylalanine—and only phenylalanine—was incorporated into a polypeptide, polyphenylalanine. Assuming a triplet code, this would mean that the message word UUU means "phenylalanine" on the level of the amino acid. This message word is transcribed from a sequence AAA in the DNA. Extensive work on the code (see Nirenberg and Leder, 86, for a recent review) has confirmed the notion that code words are triplets, and has further characterized the properties of the code to a remarkable degree. The code has also been studied in less synthetic systems, using natural, rather than synthetic messengers. Nathans and co-workers

(83) showed that a viral RNA message is translated by *E. coli* extracts into normal viral coat protein.

Regulation

Let us examine briefly the constituents of the regulatory circuitry as proposed in the gene action dogma. Jacob and Monod (59) defined the operon as a "genetic unit of coordinated transcription." It contains a cluster of cistrons, closely linked on the genetic map, each coding for a particular enzyme of a common pathway. In recent years much evidence has been produced showing that the synthesis of the enzymes coded in an operon can be coordinately induced by the common substrate, which acts as an effector. The adverb "coordinately" is the basis for the concept of the functional unit, and it calls for a master switch, the operator. In inducible strains, mutants were isolated that produced all the enzymes of an operon, even in the absence of inducer. Such mutations mapped outside the cistron did not appear to alter the structure of the enzymes, and were therefore interpreted as mutations of a regulator gene. The mutant regulator allele fails to produce a repressor; consequently, the operator is open and the operon functions. In a normal strain, regulation was thought to be controlled ultimately by effectors, metabolites of the pathway of the cognate operon. These effectors were thought to control the affinity of the repressor to the operator, which would again lead to an open or closed operon.

Very intensive research on the regulation problem has produced a substantial amount of evidence in support of the idea of coordinate control (see Ames and Martin, 6, for review). The importance and specificity of effectors was demonstrated by Attardi and co-workers (8) and by Hayashi and coworkers (52). The question to be answered was: Does the presence of inducer (effector) increase the production of a message cognate to the relevant genetic region? The answer appears to be "yes." When the synthesis of β-galactosidase was induced in an *E. coli* culture, the rate of synthesis of a specific β-galactosidase message increased, as shown by the presence of increased molecular hybridization with the corresponding DNA (52).

This result leaves little doubt that regulation can occur at the transcription level. So far, however, no one has succeeded in isolating the intermediate repressor that has been postulated in the sequence of effector-operator, although its proteinaceous nature is widely taken for granted (Jacob and Monod, 60). Furthermore, the fact that regulation can occur on the transcription level does not imply that regulation does not also occur during translation. In fact, a number of observations suggest regulatory mechanisms on the level of translation. McAuslan (76) found that protein synthesis can be regulated experimentally long after the synthesis of the cognate m-RNA is completed. Ames and Hartman (5) discuss so-called polarity mutants, which affect a structural gene of the operon and at the same time alter the rate of synthesis of enzymes cognate to cistrons distal of the mutant cistron, but not of those that are located proximal of the mutant site (toward the operator). This effect has led to the modulation concept (5), which proposes regulation of protein synthesis on the translation level, possibly involving t-RNA binding and ribosomes.

On the whole, the regulation part of the dogma is the least understood and