REGULATORY MECHANISMS IN DEVELOPMENTAL PROCESSES

Proceedings of the 3rd Symposium of the International Prize for Biology
Okazaki, 27–28 November 1987

Edited by: G. Eguchi
T.S. Okada
L. Saxén

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Regulatory Mechanisms in Developmental Processes (G. Eguchi, T.S. Okada and L. Saxén, eds.)

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Regulatory Mechanisms in Developmental Processes (G. Eguchi, T.S. Okada and L. Saxén, eds.)

Foreword

The International Prize for Biology was established in April 1985 in celebration of the sixtieth year of the reign of the Emperor of Japan. In particular, it commemorates the long devotion of His Majesty to biological research. The purpose of the prize is self-explanatory. It is to facilitate research in various fields of basic biology at the international level by rewarding a leading biologist each year. The recipients of the first two years, 1985 and 1986, were selected from eminent scientists in the field of Systematics and Taxonomy, a field in which His Majesty himself is deeply concerned.

In 1987 the recipient was selected from scientists working in developmental biology and the prize was awarded to Professor J.B. Gurdon of Cambridge, U.K. After the presentation ceremony in Tokyo on the 25th of November, 1987, the Symposium entitled 'Regulatory Mechanisms in Developmental Processes' was held at the National Institute for Basic Biology, Okazaki, Japan, with support from the Japan Ministry of Education, Science and Culture, and with additional support from the city of Okazaki. This special issue is devoted to publishing papers presented at this symposium.

A chief intention of the symposium was to discuss the results of recent studies on some key problems in developmental biology. The organizing committee of the symposium was keen to select subjects which are basic in developmental biology, but show promise of new insights afforded by investigations using new technologies and new ideas. We believed that our title would be pertinent and timely.

'Regulation' in development remains one of the most classical notions in developmental biology. In fact, this notion had been established long before the concept of regulation in biosynthetic processes. Nevertheless, the mechanism of regulation in many typical and classical examples in development, for instance, the regulative formation of twin embryos from each half of an early cleaving embryo, still remains to be elucidated. Some key processes governing complicated events of developmental regulation, however, are now becoming approachable. In this respect, it is my great pleasure to include in this issue an article by J.B. Gurdon, the distinguished laureate of the prize. He describes his challenge to solve the central, but ever puzzling, problem of embryonic induction by utilizing techniques of molecular biology which have recently become available to us.

The symposium consisted of lectures on diverse subjects, different organisms, from medusae to mammals, and different techniques, from purely embryological, such as nuclear transplantation, and physiological to molecular biology represented by gene recombination. Naturally, the potential value of transgenic systems for solving problems of developmental regulation was one of the central interests of the occasion. Nevertheless, I am confident that readers will find a good consistency in argument of each contributor. It is obvious that there is a consensus to pursue the classic subject of regulative changes in development in respect to gene expression.

This attitude, however, does not mean an effort towards the dissolution of the subject into pure molecular biology, as Gurdon stated very explicitly in his address at the Presentation Ceremony as well as in his own article in the present volume. The reason for this is clear, as essential problems of development can be properly dealt with only through studies of development per se.

The volume was carefully edited by G. Eguchi and L. Saxén, to whom I am most grateful. The present

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T.S. Okada (Okazaki, Japan)

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Regulatory Mechanisms in Developmental Processes (G. Eguchi, T.S. Okada and L. Saxén, eds.)

Address by His Imperial Highness, The Crown Prince Akihito

I would like to offer my heartfelt congratulations to Professor John Bertrand Gurdon on the occasion of his being awarded the 1987 International Prize for Biology.

Professor Gurdon is renowned for his many achievements in developmental and cell biology. One of the most important among them, I understand, was his successful transplantation, twenty-five years ago, of somatic cell nuclei from the African frog species *Xenopus* into fertilized eggs of the same species. This first successful transfer of animal cell nuclei confirmed, through the experimental techniques of cell biology, the established genetic theory that DNA expression regulates organism growth.

This groundbreaking work, I am given to understand, established a solid link between molecular and cell biology, having a major impact not only on basic biology but on its applied fields, including cell manipulation.

I take this occasion to express to Professor Gurdon my deep respect for the leading role he has played over the decades in his field of specialization.

Biology today is advancing at a rapid pace, offering wide-ranging applications in medicine, agriculture, and industry. It is through the concerted efforts and accomplishments of researchers in biology everywhere that all mankind will ultimately benefit.

In closing, let me express my hope that Professor Gurdon's continued research and leadership in the years ahead will further stimulate development in biology.

Regulatory Mechanisms in Developmental Processes (G. Eguchi, T.S. Okada and L. Saxén, eds.)

Address by Prof. John Bertrand Gurdon at the Presentation Ceremony of the 1987 International Prize for Biology,

25 November 1987

Your Imperial Highness,
Your Excellencies,
Dr. Kurokawa, Dr. Yamamura and
Members of the Committee for the International Prize for Biology,
and Ladies and Gentlemen,

It gives me the greatest possible pleasure to be here and to be able to express my very sincere gratitude for this wonderful honour. I cannot think of any award I would be more pleased to receive.

I would like to express my deep appreciation in the form of three thoughts. The first concerns the pleasure of visiting Japan. My wife is delighted to do this for the first time in her life. My own first visit to Japan took place almost exactly a quarter of a century ago in the autumn of 1962, when I had only just completed my graduate student work. I particularly remember going, at that time, to Hiroshima. I was met by Toshijiro Kawamura, a senior professor and subsequently President of Hiroshima University, and taken on a fascinating trip, lasting several days, through the interior of the country and in the inland sea. I often wonder where else but in Japan would a senior professor give up a week of his time to show a foreign student around his country. This is the kind of extreme courtesy for which Japan is famous. It left a lasting impression on me and makes me especially appreciate the honour which I now receive from this country.

Since that time, 25 years ago, Japan has undergone enormous social and cultural advances. An interesting reflection of the importance and esteem in which Japan is held in my country is seen from the fact that the school where I learnt ancient Greek and Latin, as did students for the preceding 547 years, has just now started to teach Japanese.

Another direction in which my thoughts turn at this time is towards His Majesty, Emperor Hirohito. The prize which I am so fortunate to receive is a tribute to his extraordinarily long reign. May I add my felicitations, and those of the Royal Society of which His Majesty is the only Royal Fellow who is not a member of the British Royal Family. Apart from the distinction of being by far the longest reigning monarch, he has also achieved the longest reign of any Emperor or King since the year 2187 BC when Phiops II, a Pharaoh of Egypt, is reputed to have ended a 94-year reign. Also remarkable in the history of Royalty is His Majesty's lifelong devotion to biological research, in the course of which he has published meticulous accounts of the fauna and flora which he has personally collected in Sagami Bay, Nasu, and Suzaki, and has become an acknowledged authority on Hydrozoa. For him and for Crown Prince Akihito to have carried out professional biological research in addition to performing official functions is an achievement which I greatly admire and gives special significance to this prize. The fact that this award has been given, for its first three years, to foreign scientists seems to me to emphasize its international importance.

My third thought on this occasion concerns the subject of the prize this year. My enthusiasm for

biology was initiated by collecting tiny moths formed from larvae which live in between the two sides of a leaf, but my interest soon moved to developmental biology in its own right, the subject of this year's prize. Some have suggested that the subject no longer exists, being no more than one aspect of molecular biology. My own view is different. I believe we need to combine modern biochemical methods with the kinds of embryological analysis to which Japan has contributed significantly in the past, if we are to fully understand how an egg turns itself into a complete organism. A central aspect of development is cell differentiation, and this is a field in which Japanese scientists are internationally distinguished, a situation which is, in my view, to a large extent attributable to the leadership of Professor Tokindo Okada. When I had the pleasure of first meeting him, I would never have dreamt that I would find myself here, 25 years later, receiving this pre-eminent award; and to do so in a country where he and his colleagues have made so many fundamental contributions is all the more gratifying.

May I once again express my sincerest thanks for this very great honour which I see as the highlight of my scientific career, and for entertaining my wife and myself so magnificently, in connection with this elegantly phrased 'auspicious' day.

Regulatory Mechanisms in Developmental Processes (G. Eguchi, T.S. Okada and L. Saxén, eds.)

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The origin of cell-type differences in early embryos

J.B. Gurdon

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Differences between cells first arise in embryonic development by two principal mechanisms. One is the asymmetric distribution of cytoplasmic substances at mitosis in eggs and early embryos. The other is cell interaction or embryonic induction. Certain aspects of these major mechanisms are considered, and emphasis is placed on the value of molecular markers. The effects of unequal cell division on the concentration of cytoplasmic determinants are discussed. In embryonic induction, the nature and timing of response is determined more by properties of the responding tissue than by those of the inducing molecules. Possible future directions of work are discussed in relation to experience with amphibian eggs and oocytes.

Embryonic development; Cell-type differences

Introduction

The question of how a single cell, the fertilized egg, gives rise so soon to a wide range of different cell-types is still a central problem in developmental biology. Part of the answer must be sought in terms of mechanisms of gene activation. Although the major concepts in this field have existed for many years, there are some aspects of these concepts which are not widely appreciated, and these are discussed below.

Just as interesting as the mechanisms that generate the first differences between cells are those that stabilize and maintain the differences once they have arisen. Connected with these mechanisms are the remarkable switches in cell-type from one kind to another, known as transdetermination (Eguchi and Okada, 1973; Okada, T.S., 1986). When all is understood, switches in cell-type will probably involve mechanisms similar to those that initiate the first differences between cells, but my comments below directly concern the latter.

There appear to be only two fundamental principles by which different cell-types appear in early embryos. One depends upon the asymmetric distribution of cytoplasmic materials at cell division (review by Davidson, 1986). Some cytoplasmic components are localized in the unfertilized egg, but others become localized after fertilization before the first cleavage. In yet other cases, localization takes place just before each cleavage division. In later embryonic development, especially in nematodes, asymmetric cell divisions commonly initiate divergent cell differentiation among daughter cells. This seems very likely to result from the asymmetric distribution of cytoplasmic components which activate genes. In all these examples it is assumed that cytoplasmic 'determinants' are being concentrated in certain

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cells and that these act directly or indirectly as activators or repressors of genes.

The second general principle by which cell-type differences arise is by cell interactions, or embryonic induction (review by Saxén and Toivonen, 1962). Although the phenomenon was discovered in 1901, the importance of, and interest in, embryonic induction has enormously increased in recent years. Even in organisms traditionally regarded as 'mosaic' as opposed to 'regulative' in development such as nematodes, some cell-types such as muscle are clearly formed, at least partly, as a result of cell interactions (Priess and Thomson, 1987). Also in *Drosophila* there is growing evidence that cell interactions are involved in development (e.g. Ready et al., 1976; Wharton et al., 1985). Therefore the phenomenon is more widespread in its occurrence than was originally supposed. Interest in induction has been revived recently because molecular assays have been introduced, providing much more rapid and quantitative assessments (review by Gurdon, 1987), and because defined substances, such as growth factors, have been shown to work well as soluble inducers.

Asymmetric distribution of cytoplasmic components at cell division

Three fundamental questions eventually need an answer. How are cytoplasmic substances localized? What determines the time of their action? And how do the localized components exert their specific effects?

Concerning the process of localization, the potential importance of unequal cell divisions is not always appreciated. It is the concentration of a determinant which is critical, and this must be significantly increased or decreased to become effective. If a parent cell containing a certain concentration of determinant divides into two equally sized daughter cells, it is easy to see how the concentration of determinant can be greatly *decreased* in one daughter cell (see Fig. 1B). But it can be substantially *increased* only if the volume of one daughter cell is very small compared to the other daughter. This is explained in Fig. 1. Therefore in cases where cells divide into two daughters of equal volume, gene activation seems more likely to result from a decrease in concentration of repressor than in increase in activator. The consequences of unequally sized daughter cells are also evident from Fig. 1.

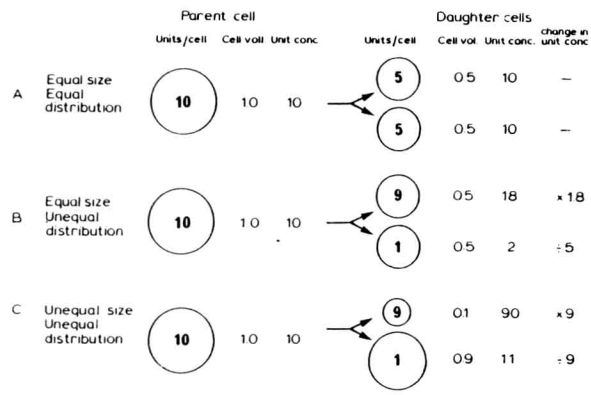

Fig. 1. Consequences of asymmetric cell division and asymmetric distribution of cytoplasmic substances (referred to as units) on the concentration of determinants in daughter cells. It is assumed, as generally happens in early embryos other than those of mammals, that no cell growth takes place after cell division. If cell division is equal, the concentration of determinants can be significantly reduced (by a factor of 5, as shown), but not significantly increased. Vol, volume. Conc, concentration.

Regarding the time of action of determinants, the simplest explanation is that they are effective as soon as a sufficiently high or low concentration is reached, that is immediately after a cell division in which they are unequally distributed. This is not a satisfactory explanation for the time of action of determinants which are localized in the 1-cell fertilized egg but which do not appear to have an effect until much later. An example is the pole plasm or germ-plasm of insects and amphibia, in which the germ-plasm, though present in the egg, has its first apparent effect (on cell division) many hours later.

One explanation for such apparent examples of the delayed expression of a determinant is that several sequential gene activations are required. A determinant would activate a gene, whose product would in turn activate another gene, and so on, until cell-type-specific genes diagnostic of specialized cells are activated. This model is popular in

Drosophila development where genes whose products seem likely to affect other genes (e.g. those with homeobox or finger domains) are activated in a temporal sequence (review by Akam, 1987). It must, however, be kept in mind that significant time is needed for a eukaryotic gene to be transcribed and its product to reach a high enough concentration to activate another gene. The rate of elongation during transcription is about 15 nucleotides per second in most eukaryote organisms investigated (Davidson, 1986). A long transcript of 100 kb would therefore take nearly 2 h to synthesize; to this must be added the time taken for splicing, transport of mRNA to the cytoplasm, translation (very commonly at 1 amino acid per s), and accumulation in the nucleus of a DNA-binding protein. It is generally observed that about 10000 copies of a protein which binds to a single DNA sequence must be present in a eukaryotic nucleus, if that sequence is to be bound in a high proportion of nuclei (Gurdon, 1985). This situation arises from the fact that a protein which binds to one sequence with high affinity (Lin and Riggs, 1975), also binds to all other sequences, of which there are a very large number in eukaryotes, with a lower but nevertheless significant affinity. It is, therefore, unlikely that in eukaryotic cells there can be a very rapid succession of entirely dependent sequential gene activations. We would suspect that the time between the first transcription of a gene and the first expression of another gene activated by the product of the first gene will be hours rather than minutes.

Another, in my view more probable, explanation of the apparently delayed effects of egg cytoplasmic determinants is that they are activated by cell interactions, which are discussed below.

On the question of how cytoplasmic determinants have their effects, only the most general comments are worth making. One apparently valid generalization is that they generally work at the level of initiating transcription. Past nuclear transplantation experiments (reviewed by Gurdon, 1986) and other work have established that genes themselves do not undergo any irreversible changes during development. There are many cases now known in which new gene expression in early development follows new transcription, rather than the translation of maternal mRNA. This is particularly clear for early ascidian development (Bates and Jeffery, 1987). Determinant molecules themselves, whether present in an egg as protein or RNA, are maternal and are not formed by zygotic transcription; but they appear to act by initiating transcription, and perhaps also novel splicing patterns, of previously unexpressed genes.

A general difficulty in analyzing the mode of action of determinants arises from imprecise assays that have to be used in some cases. An example is the pole or germ-plasm, the ultimate assay for which is fertility in an adult, but this must depend on numerous events. As has been well shown by the extensive work of M. Okada (review, 1986) and colleagues, fractions of pole plasm appear to initiate but not complete germ-line differentiation; the more convenient early assays of germ-cell differentiation do not necessarily reflect gene activity by the germ-cells. What would enormously facilitate future work is a molecular assay which specifically reflects commitment to germ-cell differentiation at an *early* stage of the process.

Embryonic induction

Three fundamental questions need to be answered about embryonic induction. One is the nature of molecular events by which a signal from one cell is transferred to another recipient cell and interpreted as specific gene expression in that cell. The second is how the timing of response to induction is regulated. The third is how the number of cells that respond to induction is controlled. These three points are discussed in turn.

In trying to trace the steps between an inducer molecule and a response, two approaches can be followed. One is to attempt the identification of an inducer molecule, and from there to identify a receptor, and so on. It has been notoriously difficult to identify inducer substances by fractionating either natural or unnatural inducing tissues though recent success has been achieved (Asashima et al., 1987). Much interest has been aroused by the discovery that previously identified growth factors can act as inducers (Kimelman and Kirschner, 1987; Slack et al., 1987), and that a

gene naturally expressed in *Xenopus* vegetal cells makes a product which resembles a growth factor and could be a natural inducer (Weeks and Melton, 1987). It is, however, important to appreciate that even with a well characterized growth factor, such as TGF-β, it can be very difficult indeed to isolate receptors. Even when a receptor has been identified, as is the case for some growth factors, it is a major task, in which no one has yet succeeded, to explain how a growth factor binding to a receptor results in a specific response. It commonly happens that a bound receptor leads to changes in cAMP or inositol derivatives, these events connecting with changes in intracellular calcium and the phosphorylation of many different proteins (Berridge, 1986). The difficulty is to proceed from this level to the activation of specific genes.

The second direction of research likely to bridge a connection between an inducer and a particular response is to work backwards from the specific activation of a gene, rather than forwards from an inducer. This approach depends critically on having a molecular marker for the inductive response. A good molecular marker is one which is expressed very soon after induction has taken place, which is quantitative, and which permits analysis at the transcript level (e.g. nuclease resistance) as well as at the level of individual cells (e.g. a monoclonal antibody), and these exist for amphibian muscle. It is my personal view that the lack of satisfactory markers has been a major obstacle hindering a molecular analysis of embryonic induction for several decades. Using muscle actin as an early marker of the amphibian mesoderm-forming induction, we have cloned and completely sequenced this gene. We have then used gene injection to fertilized eggs as a rapid assay for defining the region of the gene that is needed to respond to induction, i.e. the induction response element (Mohun et al., 1986). This turns out to be largely contained in the 250 base pairs that immediately precede the transcription initiation site of the muscle actin gene. This makes it realistic to look for proteins or transacting factors that bind to parts of this sequence.

A very desirable characteristic of any experimental induction system is that the time between

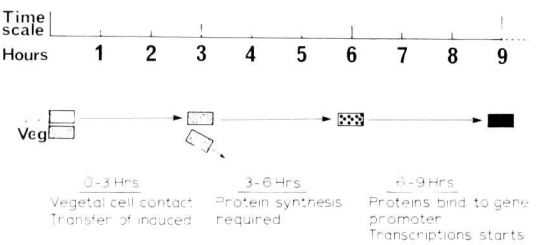

Fig. 2. Diagram of some key events by which embryonic induction initiates cardiac actin gene transcription in *Xenopus*. The starting tissues are animal (An) or vegetal (Veg) regions of a mid or late blastula. Animal cells which are not induced will differentiate as epidermis. A 3-h exposure to vegetal cells causes animal cells to express muscle genes within the following 6 h.

exposure to inducer and early response should be short. Many of the inductive systems studied in most detail have required many days before a response can be detected, and numerous events probably take place during this time (Kratochwil, 1983; Saxén et al., 1986). The muscle-forming induction which we have been investigating can take as little as 7 h (though the assay is more clear-cut at 9 h), according to the diagram in Fig. 2. As mentioned above, a significant time is required for a gene to be activated and to be able to activate another gene. It is therefore possible that there may be only one unidentified gene or group of genes that must be activated in the middle of the 7-h period, and that it is the products of these that interact with the upstream region of a muscle actin gene.

The second fundamental question concerning induction is how the timing of the response is controlled. A remarkable property of embryonic induction is that the time of response is not determined by the time when inducer is given. Using muscle gene activation as a measure of the time of inductive response, it seems that there is an internal clock by which animal cells respond at the normal time independently of when they received an inductive stimulus (Gurdon et al., 1985). Recent experiments of Grainger, Sharpe, and Gurdon (unpublished) show that the time when competence to respond to induction is lost also takes place according to an internal clock in each responsive cell. These characteristics are quite unlike

most other external influences on cells, such as responses to hormones, and emphasize the overriding importance of the processes which control responsiveness of cells, and attribute less importance to the nature of inducers and the time of their effect.

A third fundamental aspect of induction concerns the geographical control of inductive effects, that is the number and position of cells which respond. This matter is of great importance, since if too many animal cells of an amphibian blastula were to be induced to become muscle, there would be too few animal cells left to form nerve, which would then be deficient. An analysis of the factors that regulate the number of cells responding to an inductive influence has shown that several independent factors have the same effect. Thus the time when inducer substances cease to be released coincides with the time when cells lose their ability to respond to induction (review by Gurdon, 1987). This illustrates the general principle that used to be described as 'double assurance' (Spemann, 1938). It has the obvious advantage that imprecision in the timing of one of these events will generally be compensated for by reasonable accuracy of the other. It may well be a general principle of biological processes that any important event in development is regulated independently by several separate processes. This may seriously affect the interpretation of experiments. To find in an experiment that the alteration or elimination of one factor has no effect on a process, may mean only that this is not the sole factor. Each factor that helps to control a process might be altered or removed one at a time, and yet no effect observed. But if all these factors were changed at the same time, the process would fail.

Future prospects

The ultimate aim of developmental biology is to understand development in terms of molecules. As progress towards this aim starts to be achieved, it might be thought that developmental biology will cease to exist, becoming one aspect of molecular biology. Certainly techniques and concepts from molecular biology are of great value in development; but similarly, concepts and techniques originating in developmental biology are of value in other disciplines. Moreover, the processes and problems of development do not at present look like minor variants of those that operate in HeLa cells or fibroblasts. As an example, we have mentioned the remarkable phenomenon of competence or responsiveness which affects both the nature and timing of cell differentiation. Even if it turns out that the amphibian mesoderm-forming inducer is FGF or is related to it, the crucial developmental question is why blastula cells respond to FGF by making muscle, while other cells respond in completely different ways.

In respect of techniques, the manipulation and microinjection of single cells was first used successfully in large cells such as amoebae and amphibian oocytes. Gene transfer is widely used in eggs and somatic cells (e.g. Kondoh et al., 1986). The injection of messenger RNA to living oocytes has been of particular value because complex structures, such as physiological receptors, can be formed by the translation, modification, and assembly of mRNA-coded proteins. Eventually, these processes will need to proceed in cell-free systems (e.g. Suzuki et al., 1986), but living cells are proving extraordinarily useful in the meantime.

What are some of the immediate problems and how can they best be approached? It seems to me necessary to search for molecular markers of intermediate steps in complex processes. In some organisms, there is hope that mutations will reveal at least some of these steps. A most encouraging recent discovery is the frequency with which a probe for a gene product in one organism can be used to isolate a related DNA or RNA sequence in a totally different species. Even if the function of such sequences is different in various organisms, this will surely be an invaluable way of identifying important genes in vertebrates starting from *Drosophila* or nematode mutants.

There will still remain the problem of how to find embryonic substances which work at very low concentrations such as growth factors. The first growth factor to be discovered depended on finding a tissue in which it happened to be synthesized at an extraordinarily high rate (Levi-Montalcini