J. Indian Inst. Sci., Jan.–Feb. 1993, **73**, 15–30.

Developmental Biology and Genetics Laboratory

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Received on June 3, 1992.

Abstract

The Developmental Biology and Genetics Laboratory (DBG) was formally established in 1990. Its research interests fall into four broad areas: cryptic genes, transcription termination in eukaryotes, chromosomal behaviour and sex determination, and pattern formation. Even though these appear to be disparate, all of them involve the study of a common theme, namely, the facultative, heritable and reversible inactivation of a genetic unit. Here we sketch the background to the setting up of DBG and the scientific problems presently being addressed. At the end we consider possible activities for the future.

Key words: Cryptic genes, transcription termination in eukaryotes, chromosomal behaviour, sex determination, pattern formation.

1. Introduction

The Developmental Biology and Genetics Laboratory (DBG) was formally established in April 1990. To begin with it had just two staff members, both affiliated to other departments of the Institute. In two years of its functioning the strength has risen to 12. Apart from supporting staff, two others have joined the faculty, and at present (as of January 1993) we have two post-doctoral fellows and five graduate students. Our research interests fall into four broad areas: cryptic genes, transcription termination in eukaryotes, chromosomal behaviour and sex determination, and pattern formation. Even though these appear to be disparate, it is worth pointing out that cryptic genes in bacteria, heterochromatization in the mealy bug and differentiation in the slime mold share an essential feature. This feature is the facultative, heritable, and reversible inactivation of a genetic unit. In what follows we sketch the background to the setting up of DBG and follow up with a description of the scientific problems we are working on. We end by considering possible activities for the future.

2. Background

While evolution, genetics and development constitute the three threads linking our

understanding of life, evolutionary ideas, by their very nature, do not admit of ready experimental testing in the usual sense of the term. Such however is not the case with genetics or developmental biology. These have advanced in parallel with scientific experimentation more or less continuously from the days of Harvey (in the case of developmental biology) and Mendel (in the case of genetics). At the heart of both of these areas there has been the same question: how does one account for the origin of complexity starting from that apparently formless and unstructured entity, the fertilized egg? The ramifications of this question have touched all of biology.

The past few years have witnessed remarkable strides in the study of development, in particular in the use of the methods of genetics to look at developmental problems. Without minimizing the usefulness of biochemical tools in asking and answering the questions posed by development, it needs to be emphasized that there is a whole class of problems—for example, those relating to the three-dimensional shape of an organism—for which the tools of biochemistry are insufficient (D'Arcy Thompson, F. Crick). Historically, genetic (mutational) approaches have been indispensable in confronting such problems. These approaches have led to the identification of genes that appear to play comparable roles in the design of organisms as disperate as flies and frogs.

On looking back, one can discern three phases in the recent history of ideas pertaining to genetics and development. The first phase, dating from the early 1900s, is associated with the names of E.B. Wilson and T.H. Morgan. It was during this period that the existence of a deep link between the phenomena of heredity and development was first perceived. The study of this link and all its implications constitutes the area of developmental genetics. After a stage of consolidation of genetic concepts, by the early 1960s, the central problem of development came to be posed as one of differential gene expression. Namely, what is it that causes only a restricted subset of the entire genetic material to be active in any specialized tissue of the body? In addition to providing a sharpened definition of the issues, this phase witnessed intense activity on problems that had been believed to be forbiddingly difficult: specifically, those of morphogenesis, the sequence of events leading to the three-dimensional form characteristic of an organism, and patterning, the processes responsible for the stereotyped arrangement of tissues within the overall form (*e.g.*, the pattern of spots on a butterfly's wing). This came about for two reasons. On the one hand, as the result of a combined attack, both theoreticians and experimentalists came to the conclusion that Morgan's hypothesis, that graded differences in the concentrations of controlling substances might be responsible for spatial patterns, looked increasingly plausible (A. Turing, M. Locke, L. Wolpert, F. Crick). On the other hand, a systematic study of the effects of mutations in the fruit fly *Drosophila melanogaster* led to the unexpected finding that single genes could control major features of body design such as symmetry and metameric organization (C. Stern, E.B. Lewis, A. Garciá-Bellido). We are currently passing through the third phase of progress in our understanding of development. As in much of all contemporary biology, the hallmark of this phase is the molecular genetic approach. Such an approach has been made possible by technical advances that for the first time, permit

the manipulation of genetic material in a test-tube environment. The use of these techniques has already led to a knowledge of the structure of many genes of developmental interest and also to a partial elucidation of their role(s) (W. Gehring, C. Nüsslein-Volhard). It is no understatement to say that the next few years will see further revolutionary advances in our understanding of development. Making specific forecasts is hazardous, but it is clear that we will need fundamentally to change our ways of looking at the problem in order to fit even available information into a coherent picture. For example, somewhat paradoxically, the more we understand the details of gene regulation, the less plausible it appears that development is 'programmed' in the genome.

From what has been said so far, it should be apparent that at the time that we are speaking of (late 1989), the study of developmental phenomena had entered a particularly exciting phase. There was a great deal of intense activity at the Indian Institute of Science directed at contemporary problems in biochemistry, microbiology and the biology and biophysics of molecules. It was felt that the time was opportune to specifically identify developmental biology and genetics as an area of interest. Accordingly, the Developmental Biology and Genetics Laboratory was established to focus research in these areas on campus. We proceed now to give a detailed description of the problems we are working on. As will be evident, they range from purely 'molecular' ones to types familiar from classical embryology. We repeat that the justification for our working on these problems is that one goal of developmental studies is to integrate details of the regulation of gene expression with the origin of shape and form.

3. Areas of study

3.1. *Cryptic genes: their genetic and evolutionary significance (S. M. with J. Singh and M. Mukerji)*

(i) Summary

A central feature of multicellular development is that the spectrum of gene expression exhibited by any cell type is a highly restricted subset of its potential repertoire. In a curious parallel, several protozoans contain genes that can code for a functionally active product, but the genes remain unexpressed and uninducible during the normal life of the organism. Such 'cryptic' genes are an enigma in terms of their origin and evolution as also functional significance. The analogy between cryptic genes and developmentally regulated genes responsible for cell type-specific differentiation is obvious. We are using one set of cryptic genes, the *bgl* operon of *Escherichia coli*, to ask basic questions regarding the maintenance of the operon in a cryptic state. A plausible hypothesis is that there is a dynamic equilibrium between the cryptic and active states, with the active state being favored under certain environmental conditions and the cryptic state under others. We are attempting to understand the process whereby the active state becomes cryptic by characterizing Bgl^- mutants that arise spontaneously in a Bgl^+ population. The mechanisms of transcriptional activation of the operon are also under investigation.

(ii) Details

The cryptic nature of the *bgl* operon is essentially due to the presence of an inactive promoter. Though considerable information is available on the mechanism of induction of the active operon in response to the presence of beta-glucosides (this involves two operon-encoded genes), very little is known about the reasons for the silent nature of the *bgl* promoter. A major goal of our research is to understand the molecular mechanism involved in transcriptional activation of the silent promoter by DNA insertion sequences and mutations that alter DNA superhelicity. In addition to elucidating the steps involved in activating the promoter, it is of interest to address the implications of the cryptic nature of the operon in terms of the physiology of the organism. Though it has been shown that activating mutations occur spontaneously in a population at a fairly high frequency, no information is available regarding the reverse step, *viz.*, recryptification of the active operon in a population under non-selective conditions. In a natural population, a dynamic equilibrium is likely to exist between the active and the cryptic states. Therefore, any attempt to rationalize the evolution of cryptic genes must take into account the mechanisms for cryptification of the active operon.

The unique features of the *bgl* operon in *E. coli* were first demonstrated by Schaefler and coworkers[1] in a series of biochemical and genetic studies. The initial genetic characterization of the operon revealed the presence of three structural genes[1]. These studies also reported the presence of a *cis*-acting site termed *bglR* which contains regulatory elements.

Mutations that activate the operon were mapped at the *bglR* locus. The molecular analysis of the operon was started by Wright and coworkers[2]. Using recombinant DNA techniques, it was demonstrated that the major class of activating mutations contain DNA insertion sequences predominantly IS1 and IS5, located at the *bglR* site[3]. DNA sequence analysis of the *bglR* region in these mutants revealed that, in four different mutants, the sites of insertion were spread over 47 nucleotides upstream of the *bgl* promoter and CAP site[4]. Further, it was also shown that transcription always initiated from the unique *bgl* promoter downstream. Therefore, activation of the *bgl* operon was not due to the presence of a mobile promoter brought in by the insertion sequence, but by stimulation of the pre-existing *bgl* promoter in response to the presence of the activating mutations.

Several lines of evidence suggest that the functioning of the operon is not mediated *via* conventional negative regulation by a repressor. Firstly, it has not been possible to isolate recessive mutations that lead to the activation of the operon. In classical negatively regulated systems, such mutations in the repressor gene are most abundant. Point mutations within the putative operator are also expected to give a constitutive phenotype. Such mutations have not been observed even after extensive mutagenesis. Activation by insertion sequences could be considered as a result of operator mutations. However, the 47 bp minimum target region is very large compared to the average operator size of about 15–20 bp. Thus, it appears that the activation of the *bgl* operon involves mechanisms other than negative regulation by a repressor protein.

Another result of interest was the demonstration that the operon is also activated by unlinked mutations that reduce negative superhelicity of DNA[5]. This is consistent with the observation that the unlinked *bglY* gene, mutations of which were shown to activate the operon[6], is identical to the *osmZ* gene which is also involved in controlling superhelicity of DNA[7]. These results suggest the intriguing possibility that the activation of the silent *bgl* operon involves structural elements within the *bgl* promoter.

Subsequent to transcriptional activation, the operon is subjected to regulation mediated by beta-glucosides; expression of the operon occurs only upon availability of the substrate. This regulation is brought about by the products of two operon encoded genes, *bglG* and *bglF*, that act as positive and negative regulators of transcription, respectively[8]. Positive regulation mediated by *bglG* involves antitermination of transcription[9,10]. The *bglF* gene product, which is the *bgl*-specific component of the phosphoenolpyruvate-dependent phosphotransferase system, acts as a negative regulator of transcription by phosphorylating the *bglG* protein in the absence of the inducer[11]. Induction involves the dephosphorylation of the *bglG* protein. This results in its binding to the transcript near two rho-independent terminators within the operon and causes antitermination of transcription[12].

Though cryptic genes have received considerable attention, no work has been reported on them from India. Many of the questions related to the origins and evolution of cryptic genes are far from being resolved. It is interesting that a recent claim which has excited considerable attention, namely, the possibility of directed evolution, was initially demonstrated using the activation of the cryptic *bgl* operon[13]. Research in this area is in its infancy at present in our laboratory but is likely to yield interesting results.

3.2. *Regulation of transcription termination in yeast (S.M. with S. Panicker)*

(i) Summary

Unlike the prokaryotic transcriptional units in which the process of termination of transcription and 3′-end formation are well understood, the molecular events leading to 3′-end formation of the mature eukaryotic mRNA are ill-defined. In many transcriptional units, it has been shown that RNA polymerase II transcribes across the polyadenylation site and termination occurs heterogeneously at multiple sites, in some cases extending hundreds of bases downstream[14,15]. Emerging evidence suggests that in higher eukaryotes 3′-end formation is the result of processing of the pre-mRNA and concomitant polyadenylation rather than precise termination of transcription. Such processing has been demonstrated *in vitro* using synthetic RNA templates that have the 3′ processing signal[16]. The ability to edit the 3′-end precisely in higher eukaryotes relaxes the pressure to evolve an elaborate transcriptional termination system. Thus, precise termination as in the rare case of the chicken ovalbumin gene[17] is an exception. The potential for doing genetics makes *Saccharomyces cerevisiae* an ideal choice for experiments on transcription termination in eukaryotes. Deletion analysis of the 3′-end of the *HIS3* gene is expected to identify the *cis*-acting elements

involved in RNA 3′-end formation. The phenotype of these mutants is also expected to indicate the role of termination in 3′-end formation. By setting up a genetic selection for mutants that show aberrant 3′-end formation, potential candidates for genes encoding *trans*-acting elements could be identified. A detailed genetic and biochemical characterization of these mutants is expected to aid in the elucidation of the steps involved in mRNA 3′-processing in yeast.

(ii) Details

Ironically, the picture of mRNA 3′-end formation is less clear in the case of the lower eukaryotes such as yeast than in higher eukaryotes. Though recent studies have shown that 3′-end formation in yeast also involves processing linked to polyadenylation[18], these studies do not rule out a significant role for termination as an essential step preceding mRNA processing. In fact, several lines of reasoning suggest that transcription termination in yeast is likely to be significant because:

1) The intergenic spacer region in many yeast genes is much smaller than in higher organisms. In the case of the *HIS3* gene of yeast, the 3′ spacer region is only about 200 bp long. Therefore, unterminated transcription could result in interference of neighbouring genes[19].
2) Transcription of the *HIS3* gene *in vivo* by T7 RNA polymerase results in heterogeneous RNAs that are larger than the normal *HIS3* RNA (Mahadevan and Struhl, unpublished). If only processing is involved in determining 3′-end formation, the T7 transcript is also expected to be edited to give the mature mRNA of proper length. Therefore, one possible explanation is that proper processing is preceded by transcription termination.
3) There is no clear consensus for a polyadenylation site among the various yeast genes examined. Comparison of known 3′-ends does not reveal the consensus polyadenylation sequence AAUAAA encountered at the 3′ region of many eukaryotic genes. Though such comparisons have led to a proposal for a consensus sequence for yeast 3′-end formation[19], these sequences are not universally observed in all yeast genes. Therefore, 3′-end formation may be related to the formation of specific classes of secondary structures of RNA at the 3′-ends of transcripts. Formation of such secondary structures may be restricted by proper termination of transcription.
4) Alternatively, termination of transcription could be directly coupled to 3′ processing. In the model proposed by Proudfoot[15], it is predicted that the processing reaction leading to polyadenylation could leave an open unprotected 5′-end of the pre-mRNA corresponding to the 3′-end of the gene. This could be the focus of action of a 3′ to 5′ exonuclease or an RNA–DNA helicase similar to the prokaryotic rho factor. Termination of transcription resulting in the release of the RNA polymerase from the DNA template may be facilitated by such a protein moving along the RNA and encountering the RNA polymerase. Termination is likely to also involve pausing of polymerase at specific sequences downstream of the processing site. In the case of a gene containing a strong polyadenylation

signal, the processing reaction is likely to be fast and termination is expected to occur within a short distance. However, in the case where the polyadenylation signal is weak, the polymerase may travel a longer distance before the processing reaction creates an entry point for the 'terminator' protein. Thus, the direct coupling of the processing reaction to transcription termination suggests an explanation for heterogeneous termination. In addition to the processing site, other *cis*- and *trans*-acting elements may be involved in directing termination of transcription.

These observations lead to two possible molecular events in 3′-end formation:

1) Pre-mRNA processing and polyadenylation is preceded by termination of transcription. Termination at a specific site is necessary for the processing reaction by providing the correct mRNA secondary structure at the 3′-end which is recognized by the processing enzymes.

2) Termination of transcription occurs after the 3′-processing reaction and is not essential for the processing reaction. However, release of the polymerase from the template is facilitated by specific proteins that attach to the RNA after the processing reaction.

The experiments we intend carrying out are aimed at understanding the molecular events involved in mRNA 3′-end formation using the *HIS3* gene of *Saccharomyces cereviisae.*

3.3 *Genomic imprinting and sex determination (V.B. with S. Khosla)*

(i) Summary

By 'imprinting' we mean the phenomenon whereby, within the same cell or organism, the same gene, chromosome or chromosome segment functions differently depending on whether it is inherited from the mother or the father. In short, imprinting confers on the genetic system the power to encode a greater degree of phenotypic variation than is possible in its absence. Parent-of-origin effects and seemingly non-Mendelian genetic transmission are two striking consequences of imprinting. Coccids, a group of homopteran insects, make use of imprinting, meaning in this case differential chromosomal inactivation, for sex determination. We are investigating the role of DNA methylation as a correlate of imprinting in the coccids. Our experiments point to the existence of unconventional methylation at CpA dinucleotides in addition to the more conventional CpG methylation. It is of interest that complementary CpA sequences are expected to exist whenever there are $(TG)_n$ repeats (which are common in eukaryotic genomes). We are also investigating the role endosymbionts might play in sex determination in coccid insects.

(ii) Details

There are two well-known systems of facultative inactivation of chromosomes, namely, the X-chromosome inactivation in female mammals and the inactivation of the paternal set of chromosomes in male coccids. In mealybugs, embryos begin development with five pairs of chromosomes. During the 5th or the 6th cleavage

division, at which the nuclei migrate to the periphery, in some embryos the paternal set of chromosomes get heterochromatized and inactivated; these embryos develop into males. Other embryos, with both paternal and maternal chromosomes potentially active, develop into females. Such heterochromatization or chromosome condensation is closely linked to sex determination in mealybugs. The mealybugs exhibit an intermediate stage of physiological haploidy between regular diploidy and true male haploidy as seen in certain armored scale insects. This remarkable property possessed by particular chromosomes or entire sets of chromosomes, of remembering their parental origin, has been described as 'genomic imprinting'[20]. The selective inactivation of paternal chromosomes in mealybugs was convincingly demonstrated in x-ray irradiation experiments by Brown and Nelson-Rees[21]. During male meiosis, the meiotic products carrying the heterochromatic chromosomes disintegrate. Thus, it is only the maternal chromosomes in the father that are transmitted to the future generations, implying that in males, the heterochromatic set of chromosomes could undergo drastic changes (for example, post-replicative modification and rearrangements of DNA sequences) without affecting the progeny.

There have been several experiments pointing to a role for DNA methylation in X-chromosomal inactivation in mammals[22,23]. DNA methylation has also been implicated in chromosome condensation in coccids[24]. Chandra and coworkers[25,26] have shown the unusual methylation patterns of mealybug genomic DNA. In this system, methylation occurs not only at CpG sequences but also at CpA and CpT sequences. Subsequently, it was shown that a single enzyme can mediate the methylation of both of these dinucleotides within synthetic polymers[27]. Detectable methylase activity is present at high levels in males throughout development, whereas in females the levels are modulated. For instance, in females during the second instar there is hardly any detectable methylase, whereas significant levels of the enzyme are detectable during the third instar, the stage during which gametogenesis takes place[28]. At the level of DNA, apart from methylation, structural variations such as bending of DNA have been implicated in heterochromatization of mouse satellite sequences[29]. Studies with synthetic polymers have suggested that DNA methylation can enhance or induce DNA curvature[30].

Interactions of specific proteins with DNA sequences is an essential component of chromatin condensation. The protein machinery involved in heterochromatization has been revealed to some extent from the molecular and genetic analysis of position effect variegation[31,32] and regulation of homeotic gene expression in *Drosophila*[33]. Proteins such as polycomb, HP1, and suppressor of variegation (Suvar) have been shown to be involved in the global repression of genes. For instance, mutations at the Suvar (3)7 loci cause suppression of the variegation phenotype. In mammalian cells, proteins have been identified which interact with satellite sequences found in centromeric regions which are constitutively heterochromatic[34]. These observations form the components of a model proposed by Tartoff and coworkers[35]. The model proposes the occurrence of sequences acting as 'initiators' and 'terminators' of heterochromatization and postulates that any sequence translocated within this boundary is subjected to a position effect. In the light of these observations, we wish

to examine whether there is a link in mealy bugs between DNA methylation and chromatin organization mediated by specific sequence motifs and DNA-binding proteins such as those recognizing methylated DNA. Towards this we are analyzing the chromatin organization in mealybugs by micrococcal nuclease (Mnase) sensitivity. A portion of the chromatin in nuclei from male mealybugs is resistant to Mnase under conditions where the chromatin from nuclei of the female is digested completely to tri, di and mononucleosomes (unpublished observations). Preliminary experiments designed to study the nature of this chromatin fraction indicate that there is a specific sequence repeat within it along with heterogeneous sequences. We intend to clone and sequence this DNA and to study its distribution within the mealybug genome. The other aspects of chromatin condensation being pursued is the search for homologues, in the mealybug, of genes implicated in chromatin condensation in Drosophila. Among such genes are polycomb, HP1 and Suvar[31,32]. We have initiated the construction of a genomic library of mealybug DNA in yeast artificial chromosome (YAC) pYAC55 from which we intend to isolate the relevant clones.

Unlike Drosophila, the Coccids in general, and mealybugs in particular, have not been amenable to mutagenesis and genetic crosses. This is why we are interested in developing a transient assay system using mealybug embryos. In this study, reporter genes such as the bacterial beta-galactosidase gene (lac-Z) under the control of a heatshock promoter of *Drosophila* (hsp 70) are being used. When established, this system would be informative both for the study of heterochromatization as well as for our understanding of genome imprinting.

3.4. *Intercellular communication and social behaviour in slime molds (V.N. with S. Saran, R. Bhaskar and Md Azhar)*

(i) Summary

The cellular slime molds, of which *Dictyostelium discoideum* is the prototype, are ideal organisms for the study of cooperative behaviour in development. In particular, they offer a convenient system in which to examine the relative importance of predetermination ('history') and intercellular communication ('sociology') for the development of spatial patterns. When starved of food, free-living slime mold amoebae form an embryonic structure by means of aggregation. The cells in the embryo eventually differentiate autonomously to give rise to two cell types; one consists of sporulated amoebae and the other of a mass of dead cells which hold up the spores. The central problem in the study of the slime molds is: how could evolution have ever selected for suicidal behaviour on the part of the subset of cells? The same question is at the heart of the germ line-soma distinction in multicellular organisms. In both the cases, possible answers must involve individual predispositions as well as intercellular interactions as molded by natural selection. We have been engaged on a study of the distribution of sequestered calcium as a possible marker of early cellular predisposition correlated with terminal differentiation. The results obtained so far indicate that the future prestalk cells, which eventually die, have significantly higher levels of calcium than future prespore cells. Interestingly, this distinction is already

apparent at the vegetative stage, suggesting that the underlying controlling mechanism might be correlated with the cell cycle.

(ii) Details

A characteristic feature of the transition from an embryo to a differentiated larva or adult is that the state of differentiation of various groups of cells in the embryo is strongly correlated with their location. It is the feature which makes it possible to prepare fate maps[36]. Whether location and fate are merely correlated, or whether one is the cause of the other, is a central problem of developmental biology. In other words, does the fate of a cell depend on its position, or does position reflect prior differentiative tendencies? The answer to this question can vary both from organism to organism and, within the same organism, from one tissue to another. In general, the answer depends on the mechanisms for spatial and temporal control of cell differentiation operating within a particular group of cells. The cellular slime mold *Dictyostelium discoideum* is an ideal model system to study this issue: even though it involves only a small number of terminal cell types, probably just two, it exhibits all the fundamental processes underlying morphogenesis and pattern formation in higher organisms[37]. *D. discoideum* goes through a phase of multicellular development much like any metazoan embryo, with the exception that the multicellular state arises, not by repeated cell divisions, but by the starvation-induced aggregation of spatially separated cells. This makes *D. discoideum* (and other slime molds) attractive from the point of view of studying the cellular basis of morphogenesis and spatial patterning, especially in terms of the role of intercellular communication in these phenomena[37,38]. Starved cells do not divide, which means that growth and differentiation are temporally separated in these organisms. On starvation, cells aggregate at common collecting points *via* chemotaxis towards each other; up to 10^5 cells can aggregate and give rise to a multicellular embryonic structure.

The fate map of the *D. discoideum* slug consists of two spatially segregated cell types. Roughly the anterior 15% of the slug consists of prestalk cells (which die) and the posterior 85% of prespore cells (which become spores and so survive)[39]. A small minority of non-prespore cells is present in the posterior region of the slug. These are called, because of their staining properties, 'anterior-like' cells[40]. Cells located at the rear-most part of the slug, the rear-guard cells, also die and eventually make up the basal disc of the fruiting body[41]. There is a regular interconversion among anterior prestalk cells, anterior-like cells and rear-guard cells[42], and despite the difference in their localizations, these cells seem to constitute basically the same cell type with very minor differences[43].

Maeda ande Maeda[44], using a combination of atomic absorption spectroscopy and alizarin red staining, concluded that more calcium was present in the prestalk region than in the prespore region. Tirlapur *et al*[45] and Saran and colleagues (unpublished) have shown that there is predetermination at the post-vegetative stage, after which like cell types sort out to give the normal pattern of morphogenesis. Recent data[46] give clear evidence of the existence of two functional classes of amoebae at the vegetative state itself. Vegetative amoebae when stained with chlortetracycline, a

fluorescent probe used for the measurement of sequestered calcium, fall into a bimodal distribution depending upon the level of fluorescence. Little or no fluorescence is seen from prespore cells while high levels of fluorescence are seen in prestalk cells. We interpret these findings to mean that sequestered calcium levels foretell cell fate. Calcium could play a causative role in the differentiation of cell types, with high calcium marking out future stalk (and basal disc) cells. Causation could be direct: there is some indication that calcium by itself can induce stalk cell differentiation[47]. On the other hand, calcium could exert its effect indirectly. Calcium levels are known to oscillate spontaneously[48,49]; if these oscillations also exist in the slug, they could subserve a system of intercellular communication *via* wave propagation[50] which in turn could be used to establish a spatial 'map' within the slug.

The question we are left with is: if vegetative cells differ phenotypically to the extent that they appear to do, what might be the cause? An obvious possibility that suggests itself is that the cell-cycle stage at which an amoeba is starved may influence its fate, and this conjecture is supported by older results[51,52]. We have with us an S phase-specific monoclonal antibody[53] and we hope to use this antibody together with CTC in double-labeling experiments in order to look for a correlation between cell-cycle phase and sequestered calcium. A long-term goal is to investigate the link between calcium, the cell cycle and cell type-specific gene expression.

4. How we see our future

4.1. *A shift in paradigm?*

Ever since the pioneering days of T.H. Morgan and his school, the dominant guiding principle in the study of development—even if not always stated as explicitly—has been that genes 'program' development. Thus, it has been taken for granted that in order to understand development it is sufficient to catalogue genes of developmental interest and their interactions. Underlying this approach is the belief that there is a logical, perhaps algorithmic, structure to development. It is clear that one cannot adopt such a point of view and at the same time continue to think of plants and animals as having been shaped by the purely opportunistic forces of natural selection. Quite apart from this basic difficulty, recent advances have shown that it is unlikely that there are genes whose sole purpose is to guide development. Instead, what is probable is that pre-existing gene products have been coopted during evolution to fulfil new roles, some of them 'developmental' roles, as and when the need arose. For instance, a mutation in a gene involved in melanin biosynthesis can cause very specific and characteristic defects in the visual pathway; an adenyl cyclase mutation can speed up the tempo of development. Findings from recent studies on molecular evolution are consistent with the new viewpoint but cannot be easily accommodated within the conventional, programmatic way of looking at development. For example, one sees that major morphological changes can occur in evolution without large-scale genetic changes (genetically speaking, the caterpillar and the butterfly are identical). Conversely, major genetic changes do not necessarily lead to morphological novelty.

Results such as these are leading to a non-conventional point of view (granting

that this is still a minority school of thought). The shift in emphasis can be expressed as one from genes to gene products, or from nucleus to cytoplasm, or, put most concisely, from genotype to phenotype. This alternative approach suggests that development, considered as a product of evolution, should be visualized as a dynamic process: the same genes and the same set of gene products can lead to qualitatively different outcomes depending on initial conditions (*e.g.*, maternally specified information, or even environmental variables) and spatial and temporal interrelationships. If valid, such an approach would require that, new research strategies should be initiated for the study of developmental problems. Among other things, this would involve the study of cellular and genetic *networks*.

4.2. *Directions*

The power, and ease of use, of modern molecular biological techniques have made it possible to tackle developmental phenomena at a level not imagined even a decade ago. One result is that developmental biology has become an intensely competitive and rapidly advancing field. Given this state of affairs, it would be unrealistic for us to try to match our efforts with those of the lavishly endowed laboratories of the West when it comes to problems demanding 'high technology'. Instead, while keeping up with technical advances, we intend to choose systems, problems and approaches with particular care. The essence of development lies in two features: embryos change their form, and the change involves a succession of time-dependent events. It is this inescapable temporal backdrop that marks the main difference between a *developmental* study and any other study of (say) control of gene expression. In addition to the lines of research sketched earlier, the following points will be kept in mind as guides for future decisions:

(a) The recent past has seen a renaissance in microscopy. Today, at the level of the light microscope, it is possible to visualize details of intracellular contractile filaments; using fluorescence microscopes and image analyzers, one can reconstruct the spatial and temporal architecture of the living cell at resolutions of < 1 μm and < 1 second, respectively. This is a 'low-technology' area but one in which judicious investment right now is capable of yielding rich dividends in the foreseeable future.

(b) We are still at a stage of formulating theories of development and in such a situation almost any new result will be significant. Biology is regarded as an experimental science, and purely theoretical approaches are unlikely to be fruitful. What one needs is the right combination of theory and experiment. We list here what appear to us to be interesting problems for us to tackle:

   (i) Mechanical forces are believed to play an important role in the development of form and shape, but how they are organized and coordinated is poorly understood. Certain experiments suggest that there may be significant local autonomy in the expression of the forces leading to tissue movement and specific contacts (*e.g.*, exogastrulation). How are these forces regulated and coordinated?

(ii) The spectacular success of 'wet' biochemistry and molecular biology, and a philosophical attitude favoring the steady state ('homeostasis'), have tended to obscure subtle but important features of living systems, in particular their temporal organization. Evidence for calcium oscillations, with periods in the range suitable for subserving developmental roles, is available from a number of systems. The consequences of temporal patterning in intercellular signaling needs to be explored.

(iii) While it is believed that somatic development does not entail irreversible genetic changes, as we have seen earlier, the existence and potential importance of reversible modification of DNA (*e.g.*, imprinting) is being increasingly appreciated. Reversible modifications offer, firstly, an obvious means of generating variant phenotypes within the same heritable genotype and, secondly, of mediating the influence of the environment on development. Explicit models for environment–genotype interactions would make possible a better understanding of developmental canalization and of the role of canalization in evolution. The conventional explanation of canalization is that it involves selection at modifier loci, *i.e.*, just the sort of loci expected to regulate imprinting.

(iv) The functioning of genetic networks has to be examined keeping evolution in mind. Studies on randomly connected Boolean networks have shown that given certain assumptions, a system made up of elements, each receiving two inputs and delivering one of two outputs, can exhibit stable patterns of cyclic behaviour; each cycle can be identified with a differentiated cell type. The system begins to exhibit undesirable features as the number of inputs is increased beyond two and this is almost certainly so because in the model[54] connections are made at random. Also, individual cycles do not appear to have any life of their own. It would be interesting to study these networks as they evolve starting from a collection of a small number of elements. Cellular automaton models might offer a practical route to building an 'embryo'.

India has a rich pool of quite unexplored flora and fauna. To take just three examples, insects, amphibians and reptiles have been identified as groups of interest for other reasons (*e.g.*, as pests, for the conservation of biological diversity, for maintaining the stability of ecosystems). Both groups include species on which genetical and developmental studies have been meagre; it is certain that they will yield entirely unexpected insights into how animals develop. We intend to pursue studies focussed at the level of whole organisms. These would include the interrelated areas of genetics, physiology (including neurobiology) and behaviour. Traditionally, biology has meant study of particular plant or animal systems; what we should plan for is the creation of independent groups of scientists engaged in the study of the same organism from very different points of view. Past history suggests that this is not an unrealistic goal.

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