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The works summarized in this publication were presented by their authors at a Symposium held on 10th November 1987 at the Fundación Juan March.

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Genetic Strategies in Development

Symposium in honour of Antonio García Bellido

Severo Ochoa
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Fundación Juan March

Serie Universitaria

240

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OPENING ADDRESS

SEVERO OCHOA
Premio Nobel de Medicina 1959
Centro de Biología Molecular
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IN HONOUR OF DOCTOR ANTONIO GARCIA- BELLIDO

Some time ago, a number of friends, colleagues, and students of Antonio García-Bellido thought of organizing an international Symposium to honour him -- the person and the scientist. The participants would be leading figures in the world of biology who had made outstanding contributions to advances in Developmental Biology, both in its genetic and molecular aspects.

Developmental Biology is a new field with virgin frontiers and unknown territories. Men like García-Bellido have contributed brilliantly to knowledge in this area, and his work has sparkled the enthusiasm and admiration of many biologists.

García-Bellido's achievements, like those of Cajal, have been recognized everywhere, regardless of national frontiers. All of his friends have welcomed his success. His international reputation has been consolidated by his election to such institutions as distinguished as the Royal Society, the U.S. Academy of Sciences, and the American Academy of Arts and Sciences. García-Bellido is often called upon by European and American universities and research institutions to give lectures and seminars.

We, his friends, believe we should render him the honour he so fully deserves and, on invitation by the Fundación Juan March, have organized an International Symposium under the general heading "Genetic Strategies in Development", with participation of men like Sydney Brenner, Eric H. Davidson, John B. Gurdon, David S. Hognees, François Jacob, Edward B. Lewis, and Gunther S. Stent.

The programme is as follows:

BRENNER, Sydney	"Molecular genetic analysis of development in C. elegans"
STENT, Gunther S.	"Cell lineage and segmentation in development"
LEWIS, Edward B.	"The bithorax strategy"
HOGNESS, David S.	"Regulation of homeotic gene expression in drosophila"
DAVIDSON, Eric H.	"Maternal and zygotic DNA binding proteins required for spatial activation in the sea urchin embryo"
GURDON, John B.	"Embryonic induction"
JACOB, François	"Retroviruses and early stages of mouse embryo"

We in Spain are proud that one of the main branches of modern biology should have found such a strong base in this country as García-Bellido has secured for Developmental Biology and Genetics.

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MOLECULAR GENETIC ANALYSIS OF DEVELOPMENT IN *C. ELEGANS*

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The complete structure of the nervous system of C. elegans has been determined by reconstruction of serial section electron micrographs. Since the developmental cell lineage is also known, this means that we have a complete description of the position and origin of every cell, and, in the case of the nervous system, of all the interconnections. The task now is to map this onto the genome of the organism, by the analysis of mutants.

Specific genes can now be readily cloned from the nematode. Extensive use is being made of transposon mutagenesis, and linkage to DNA polymorphisms, many of which are due to inserted DNA. A powerful aid has been the construction of a physical map of the nematode in the form of an ordered library of cosmid clones. The ease with which new genes can be found by linkage alone will increase as more genes are placed on the map.

The first mutants isolated in the nematode included a large set which affected the movement of the animal. A subset of these were early recognised to affect the body musculature and a number of these have been traced to the molecular level. Thus unc-54 is the structural gene for the heavy chain of a myosin specifically expressed in body muscle cells while unc-14 specifies paramyosin, another component of the thick filament.

The majority of the unc mutants affect the nervous system. Many years ago we studied the anatomy of a small selected set of these mutants and found changes in several of them but could not interpret the results because we did not have the complete anatomy of the nervous system. J. White and others have now returned to this work and have found very specific changes in the anatomy of the motor nervous system in a number of these mutants. The genes for these are now being cloned and it will be fascinating to see what insights we will gain into the construction and functioning of this simple nervous system.

This approach is now the standard one followed by many workers pursuing the molecular basis of complex biological processes. Some have referred to it as reverse genetics but it is really reverse biochemistry. It relies on our ability to clone and sequence DNA, and also on our capacity to interpret the sequences. The former is well advanced, the latter, less so; and as time goes on and the amount of raw sequence data grows, it will become the limiting factor in our understanding.

CELL LINEAGE AND SEGMENTATION IN DEVELOPMENT

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Several protostomal as well as deuterostomal phyla, such as the annelids, arthropods and vertebrates, share the general structural feature of their bodies being composed of a periodic series of bilaterally symmetric segments. Each segment corresponds to a module, or metamere, of regularly iterated morphological elements, such as appendages, skin specializations, muscles or nerve cell ganglia. This basic morphological segmentation pattern is often obscured, however, because the metameres, rather than being exactly alike, usually differ at various positions along the longitudinal body axis, from head to tail. My presentation will focus on the role of cell lineage in the development of body segments in leeches (Hirudinea). Their tubular body consists of 32 longitudinally differentiated segments and a non-segmental prostomium. The metameric morphological elements include the segmental ganglion of the ventral nerve cord, a bilateral pair of excretory organs, or nephridia, and three subdivisions of the skin, or annuli. One of these three annuli lies in register with the segmental ganglion and includes circumferentially distributed sensory organs.

Development of the fertilized leech egg proceeds via a stereotyped sequence of (holoblastic) cleavages, giving rise to an embryo whose cells can be identified individually by various criteria, including size, position, birth order and cytoplasmic specializations (Figure 1). Five large bilaterally paired blastomeres, designated teloblasts, undergo a series of iterated, highly unequal divisions, and generate five paired bandlets of several dozen much smaller primary blast cells, designated m, n, o, p, and q. Ipsilateral bandlets merge to form left and right germinal bands and the two bands migrate over the surface of the embryo and eventually coalesce in rostrocaudal sequence to form a sheet of cells, the germinal plate, along the future ventral midline.

Proliferation and differentiation of the blast cell clones gives rise to a morphological periodicity of the germinal plate, reflecting

formation of the 32 body segments. Within the germinal plate, the clones founded by the older blast cells of each bandlet lie anterior to the clones founded by the younger blast cells. Hence throughout most of its development, the germinal plate embodies a caudorostral developmental progression, according to which the development of a given segment is more advanced than that of its next posterior segment. Cell proliferation also causes the germinal plate to expand laterally around the circumference of the embryo, until the right and left leading edges of the plate meet along the future dorsal midline. At this point, formation of the body tube of the leech is complete.

Use of novel cell lineage tracer techniques to mark the differentiated post-mitotic descendants of individual blast cell bandlets has shown that each primary blast cell gives rise to a metameric hemilateral kinship group of a few dozen segmentally iterated, identified cells. Either side of each segment was found to comprise one mesodermal kinship group (including muscle fibers and nephridial cells), clonally derived from a single primary m blast cell, and six distinct ectodermal kinship groups (including central and peripheral neurons, as well as epidermal cells), of which four are derived from two serially successive primary n and q blast cells and two from one primary o and one primary p blast cell. Each of the seven hemisegmental kinship groups arises from its founder blast cell via a clone-specific, stereotyped cell lineage pattern. Specific photoablation experiments have shown that the stereotypy of these patterns is (largely) an autonomous property of individual blast cells, rather than being the product of intercellular interactions. An exception to that rule is presented by the o and p blast cells, of which one can take on the fate of the other and whose commitment to one of two alternative developmental fates does depend on intercellular interactions.

The differences in segment morphology along the longitudinal axis of the leech body are reflected in corresponding modifications of the cell lineage pattern to which serially homologous blast cell clones give rise. These modifications do not seem to be attributable, however, to intrinsic differences between serially successive primary blast cells -- for instance to the rank of their birth -- as can be inferred from experiments in which slippage of a blast cell bandlet has displaced a primary blast cell to a segment inappropriate for its

birth rank. Under these conditions, the displaced primary blast cell gives rise to a lineage pattern appropriate for its ectopic position rather than its birth rank.

The segmentation in insect larva bears considerable morphological resemblance to that in the phyletically related leeches. However, despite this similarity -- in fact, undoubted homology -- the early course of embryogenesis in flies is radically different from the stereotyped sequence of holoblastic cleavages encountered in leech development. The fly egg begins its development with a series of synchronous mitotic divisions onwards from the zygote nucleus, unaccompanied by cell division, giving rise to an embryonic syncytium containing thousands of nuclei. Eventually most of these nuclei migrate to the periphery of the syncytium, where each nucleus becomes cellularized by an infolding of the embryonic cell membrane. In this way the fly embryo comes to consist of a uniform sheet of about 6000 cells, the cellular blastoderm, none of which has actually arisen by cell division. It is only in subsequent embryogenesis that cells of the blastoderm proceed to divide and generate cell clones.

Since the concept of cell lineage is not applicable to fly embryogenesis before formation of the approximately 6000 cells of the blastoderm, here the origin of segments cannot be traced back, as they can be in the leech, to the serial production of homologous sets of segmental founder blast cells by iterated divisions of a few paired teloblasts. Rather, it would appear that in the fly segments arise by subdivision of the two-dimensional sheet of blastoderm cells into a longitudinal series of 15 or more circumferential bands, of which each comprises a set of a few dozen founder cells committed to the formation of one segment. This subdivision process appears to be effected by the hierarchical interaction of the products of a special set of a dozen or more genes.

Accounting for the evolutionary divergence of the mechanisms of embryogenesis in the two phyletically related taxa, flies and leeches, presents developmental biologists with a challenging problem.

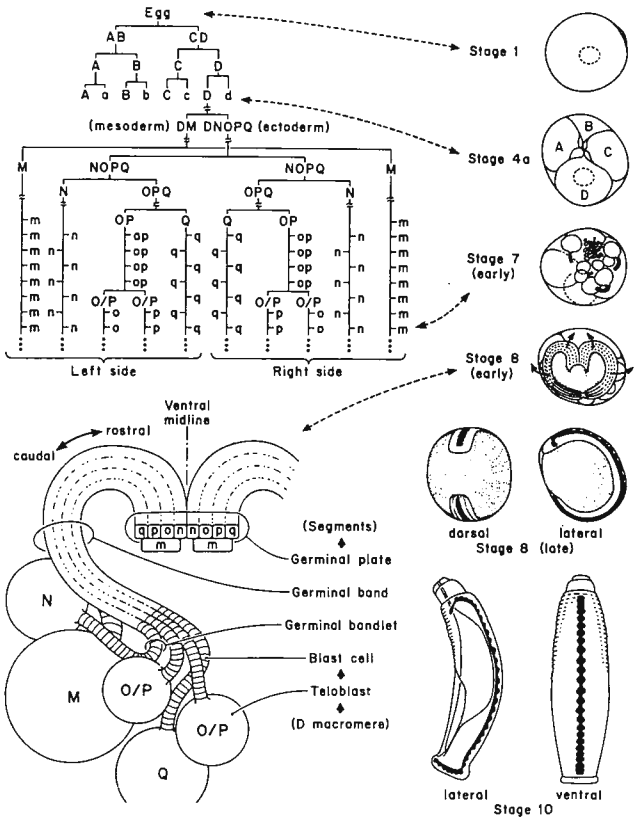

Figure 1. Schematic summary of the development of the leech. Upper left: cell pedigree leading from the uncleaved egg to the teloblast pairs, M, N, O/P, and Q; and the paired primary m, n, o, p and q blast cell bandlets. Lower left: hemilateral disposition of the teloblasts and their primary blast cell bandlets within the germinal band and germinal plate. Right margin: diagrammatic views of the embryo at various stages. In the stage 8 (early) embryo, the heart-shaped germinal bands migrate over the surface of the embryo in the directions indicated by the arrows. In the stage 8 (late) embryo the germinal plate is shown to lie on the ventral midline, with the nascent central nervous system and its ganglia indicated in black. In the stage 10 embryo shown, body closure is nearly complete. The prostomium is derived from blastomeres a, b, c, and d.

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THE BITHORAX STRATEGY

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In Drosophila it now seems clear that once the fundamental segmentation plan of the embryo is laid down the next stage involves programmming the differentiation of the body segments into head, thoracic and abdominal regions. This programming appears to be elegantly accomplished by two gene clusters: the Bithorax (BX-C) [1] and Antennapedia (ANT-C) [2] gene complexes. The BX-C and ANT-C lie in the right arm of the third chromosome and although separated from one another there is now evidence based on DNA sequence homology among the homeobox-containing genes of these complexes that the complexes themselves may have arisen from a common ancestral gene or genes which underwent tandem duplication and mutational divergence, eventually breaking up into the two complexes. The discovery of the homeobox [3;4] and the demonstration that it is indeed involved in binding to DNA [5] supports the assumption that the BX-C genes "produce a whole set of new substances that repress certain systems of cellular differentiation and thereby allow other systems to come into play" [6] -- an assumption that now appears too restrictive since the BX-C genes may also activate such other systems directly. The discovery of two clusters of genes in mice [7] and human beings [8] having homeobox sequences that are remarkably conserved relative to those of the ANT-C and the BX-C suggests a common theme to the developmental strategies followed by the vertebrates and invertebrates.

The BX-C genes are all related to one another in function in the formal sense that a loss-of-function of a given gene results in one region of the body being transformed toward a more anterior region; for example the type mutant, *bithorax*, *bx*, results in the anterior portion of the third thoracic (T3), or haltere-bearing, segment being transformed toward T2, the wing-bearing segment. Fortunately the wild-type function of many of the BX-C genes does not have to be inferred indirectly from the behavior of mutant alleles, but instead can be determined directly by examining the effects on embryonic development of having only a subset of the BX-C genes present. The analysis [1] is made possible by the fortunate circumstance that although an animal lacking all of the genes of the complex (homozygous for the deletion known as *Df-P9*) dies, it completes embryonic development and has the body segments commencing with T3 and extending posteriorly (at least to A7) transformed toward a T2 level of development (LT2) -- the latter being the ground state with respect to BX-C functions. If now a duplication for essentially only the wild-type *Ultrabithorax*, Ubx^+, region of the complex (*Dp-100* in 3L) is added to the homozygous *Df-P9* genotype then segments commencing with T3 and extending posteriorly transform from LT2 toward LT3. If *Dp-100* is replaced by a somewhat larger duplication, *Dp-P10*, that contains the wild-type *bithoraxoid*, bxd^+, as well as Ubx^+ regions then segments commencing with A1 and beyond transform from LT3 toward LA1. Hence the adjacent transformations LT2 to LT3 and LT3 to LA1 are controlled by the adjacent Ubx^+ and bxd^+ regions, respectively. The situation is somewhat more complicated in that the unit of body segmentation that the BX-C genes actually transform tends to be the fundamental body segment, or parasegment [9] -- the observed body segments being derived by fusion of posterior and anterior halves of adjacent parasegments.

The bithorax complex is known to extend over nearly 400 kb [10;11] and to contain three homeobox regions located in the *Ubx*, *infra-abdominal-2*, *iab-2*, and *iab-7* genes,

the latter two being known also as the *abd-A* and *Abd-B* genes, respectively [12]. How the BX-C genes control much of the development of the body plan commencing within the second thoracic segment (T2) and extending posteriorly may be thought of as the bithorax strategy. This strategy might be likened to an assembly line (the chromosome) along which the operators (the BX-C genes) sequentially assemble a complex machine consisting of many sequentially arranged parts (the segmented organism). The strategy is a dual one and involves (1) regulation of the BX-C genes themselves so that they turn on in the right place at the right time and (2) regulation by the BX-C genes of downstream functions carried out by still other genes outside the complex. Garcia-Bellido has designated the latter as "realisator" genes [13] on the basis that they realize a destiny imposed upon them by "selector" genes, i. e., genes of the BX-C or ANT-C type. He and his colleagues are already engaged in trying to identify and characterize specific realisator genes in relation to their selector genes [14].

Only a few of the properties governing regulation of the downstream functions will be cited here: (1) the BX-C genes appear to control cellular specificities as shown in experiments involving reaggregation of imaginal disc cells [15] (2) many of the BX-C genes seem to share some functions in common such as establishing the connections of the tracheal trunks of the larva [1] or controlling formation of specific types of neuronal structures [16] and (3) in addition to sharing some functions in common, certain genes seem to have unique functions; thus, only the *bxd* gene seems capable of suppressing development of ventral pits on the abdominal segments and only the *iab-4* gene appears to be able to activate development of the somatic gonad [1]. In none of these cases do we have any knowledge of what realisator genes may be involved.

The regulation of the BX-C genes themselves involves both cis and trans controls. Four classes of genes are known to regulate the BX-C in trans; (1) numerous genes acting negatively, e. g., *Polycomb*, *Pc* [1;17] *extra sex combs*, *esc* [18], and *Polycomb-like*, *Pcl* [19]; (2) three genes acting positively: *Regulator of bithorax*, *Rg-bx* [20], with a recessive allele, *trithorax*, *trx* [21], and two genes, symbolized as *ash-1* and *ash-2*, that appear to resemble *trx*, but are not allelic to it nor to each other [22]; (3) certain segmentation genes that seem to interact with the BX-C possibly via the homeobox domains, such as the control of the BX-C by a gene within the ANT-C, *fushi tarazu*, *ftz*, [2;23] as shown genetically [24] and molecularly [25] and (4) cross-regulation by genes within and between the BX-C and ANT-C, possibly also mediated by homeobox regions [26;27;28].

We are concentrating on the cis regulation of the BX-C genes since several unusual phenomena are involved. Such regulation obeys a number of rules [29] (1) the cis-inactivation (CIN) rule states that a mutant lesion within most if not all of the genes of the complex tends to produce a partial to complete loss of function in one or more genes located immediately distally; (2) the cis-overexpression (COE) rule states that mutant lesions within a subset of the BX-C genes tend to produce a gain of function of the gene located immediately proximally; interestingly this subset of genes does not include the three known homeobox-containing genes of the complex; and (3) the colinearity (COL) rule states that the order of the genes in the chromosome parallels their order of expression along the body axis.

Two cis-operating mechanisms can be imagined to account for the precise regulation of the BX-C gene functions along the body axis: it has been suggested [1] that the BX-C genes become derepressed in accord with a proximo-distal gradient along the

chromosome in the relative affinity of cis-regulatory elements for repressor molecules; in addition some type of cis-acting entity seems needed to account for the CIN and COE rules; such as, anti-sense RNA, or regulatory RNAs [29;30;31]. Although by definition proteins would seem ruled out as cis-acting entities, DNA-binding proteins might be able to function more efficiently in cis than in trans. A model [29] which attempts to provide a common mechanism for the CIN and COE effects and the related phenomenon of transvection [32] invokes a cis-acting entity that tracks the chromosome and is able to a limited extent to interact with the BX-C in the homologous chromosome provided somatic pairing is not disrupted. It has also been proposed that the non-homeobox-containing genes; e. g., *bxd*, act like enhancers [11;30;31].

In the unravelling of the bithorax strategy and other developmental strategies we can safely predict that there will be many surprises still to come and that Garcia-Bellido and his many colleagues and students will be furnishing us with many of these surprises.

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REGULATION OF HOMEOTIC GENE EXPRESSION IN *DROSOPHILA*

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Looking back at the significant developments that have placed Drosophila melanogaster in such a conspicuous position respecting its contributions to our understanding of development, one is struck by their genetic bias. I would argue that this is because the genetic approach is ideally suited for sorting out the important elements in such a complex process as development. That argument is nicely supported by the genetic dissection of the development of the metameric pattern that characterizes the anterior-posterior axis of the Drosophila body plan.

Lewis's work on the homeotic mutations defining the bithorax complex (1) and Garcia-Bellido's work on compartments (2) are cases in point, for they indicated that the establishment of metameric identity is a discrete step, distinct from the formation of metameric boundaries, and specified by a discrete subset of the genes. The general concept that the metameric pattern is generated in discrete steps specified by functionally distinct subsets of genes derived primarily, however, from the work of Nusslein-Volhard and Wieschaus (3). Their classification of mutations altering the metameric pattern in embryos according to the kind of alteration they elicit, yielded a surprisingly simple result - namely, a small number of discrete kinds of defects resulting from mutations in a correspondingly discrete class of genes. Thus, these segmentation genes can be divided into three functional classes according to three kinds of alteration their mutations induce. One kind deletes pattern elements corresponding to several adjacent segments and defines the "gap" class of genes. The other two kinds of mutational alterations of pattern are periodic. One kind affects the pattern with a period equivalent to two segment lengths - deleting, for example, every other segment boundary and reducing the number of segments by a factor of two. The corresponding genes are therefore called "pair-rule" genes. The other kind exhibits a period of one segment length and defines the "segment polarity" class of genes. The zygotic genes contribute to each of these three classes. Curiously, however, maternal effect genes contribute only to the gap

genes - an observation that would indicate that the gap class precedes the two periodic classes in a temporal series. Maternal effect genes also contribute to a class of "polarity genes" characterized by mutations that disrupt global polarities in the embryo.

The most logical temporal series for these four classes is polarity genes followed by the gap genes, followed by the pair-rule and segment polarity genes. The homeotic genes would then follow the periodic segmentation genes. A better hierarchal ordering requires a knowledge of the gene products, which in turn allows one to determine the effects of mutations in one class of genes upon the expression of another class. This knowledge came from parallel developments in Drosophila molecular genetics, many arising from work in our laboratory; namely, the ability: (i) to construct libraries of cloned genomic DNAs (4); (ii) to identify clones containing sequences homologous to those in a given RNA or DNA (5); and (iii) to isolate genes defined only by their mutations, using, for example, the techniques of "chromosome walking and jumping" (6), which led to the first isolation of homeotic genes - specifically the Ubx domain of the bithorax complex (7).

Other homeotic genes have since been cloned, as have many of the segmentation genes (8). The RNA and protein products of a growing number of these have been defined, allowing determination of the spatial distribution of these products in both wild-type and mutant animals. Experiments of this sort have begun to yield hierarchical models for the genetic regulatory interactions among members of a given genetic class and between members of different classes (for examples, see reference 8) - models that are in general accord with the temporal series noted above. Furthermore, these models suggest that the spatial resolution of pattern information is refined by regulatory interactions among genes in a given class, and that the result of that refinement may be passed down the hierarchy to the next class by the products of only a fraction of the genes within the class.

These hierarchical models are, however, incomplete. In particular, the procedures from which they derive cannot generally determine if the requirement of one gene for the appropriate expression of another results from direct regulatory action of the protein(s) encoded by the first gene upon the second. Such a determination typically requires the isolation of the proteins in question and the characterization of their genetic regulatory properties. We are at this stage in our studies of the Ubx transcription unit of the

bithorax complex, in respect both to the regulatory properties of the Ubx-encoded proteins and to the regulation of the Ubx unit by proteins encoded by other homeotic genes and by pair-rule segmentation genes. In this talk, I shall focus on our current knowledge of these regulatory problems after briefly summarizing the pathway by which we have moved from the formal genetic to the molecular genetic, and now to the biochemical definition of the Ubx homeotic functions.

The molecular mapping of a large number of mutations within the Ubx domain and the definition of its transcription units and their RNA and protein products led to a significant simplification and change in our concepts of its organization over that derived from the formal genetic studies (9). Instead of the multiple distinct genes postulated from the properties of the abx, bx, bxd, pbx and Ubx mutations, the molecular genetic results demonstrate that the genetic functions indicated by these recessive mutations can be accounted for by the proteins encoded by a single long 78 kb transcription unit (Ubx unit) that is coextensive with the region where the Ubx mutations map, and by cis-regulatory elements located where mutations in the other four classes map - either upstream from the Ubx unit in the 40 kb bxd region (bxd and pbx mutations), or within the largest intron of the Ubx unit (abx and bx mutations).

We have shown that alternative splicing of the Ubx transcript yields five mRNAs whose relative abundance varies during development. The Ubx proteins they encode contain from 346 to 389 residues and are characterized by N- and C-terminal constant regions joined by a variable region consisting of different combinations of three elements: I, II and III (in N-to-C order) comprising 9, 17 and 17 residues, respectively. Of the 100 residues in the C-terminal constant region, 60 comprise the homeo domain encoded by the Ubx "homeo box". This domain is proximal to the variable region which may thereby modulate its DNA-binding function.

The Ubx proteins have been overexpressed and subsequently purified and examined for their DNA-binding properties at sites associated with two homeotic genes. One of these is the Ubx gene itself, whose positive feedback regulation had been suggested by the maintenance of its position-specific expression in the period following the early transient expression of certain segmentation genes (e.g., the pair-rule gene ftz) that initially determine Ubx expression. The other is Antennapedia (Antp), a homeotic gene which, on the

basis of mutational data, appears to be repressed by Ubx products. Several binding sites in the DNA associated with these genes have been identified. Their consensus sequence, at least for one member of the Ubx protein family, is $(TAA)_5$.

An in vivo cotransfection assay in Drosophila cultured cells has recently been developed to examine the regulation of these genes by Ubx proteins. In this assay, one of the two cotransfected plasmids produces the Ubx protein, while the other contains the promoter region of a given gene fused to a CAT (chloramphenicol acetyl transferase) reporter gene so that the effect of the Ubx protein on the promoter can be determined by measuring the CAT activity. We have thereby shown that Ubx proteins directly regulate the Ubx and Antp genes in positive and negative manners, respectively. Similarly, we have shown that the ftz protein positively regulates both the Ubx and Antp genes. Modification by in vitro mutagenesis of the Ubx proteins and of the promoters used in this system is in progress to define the protein domains and the DNA binding sites relevant to the observed regulation.

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MATERNAL AND ZYGOTIC DNA BINDING PROTEINS REQUIRED FOR SPATIAL ACTIVATION IN THE SEA URCHIN EMBRYO

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This presentation will concern the molecular basis of the process by which differential patterns of regional gene activity are instituted in the embryo. The cytoskeletal actin gene CyIIIa provides an excellent molecular marker for the zygotic program of gene expression characteristic of the embryonic aboral ectoderm. According to our recent lineage tracer studies the aboral ectoderm derives clonally from 6 specific cleavage stage founder cells. An additional marker gene considered is the SM50 spicule matrix gene, expressed exclusively in skeletogenic mesenchyme cells, the clonal 5th cleavage descendants of the micromeres. Regulatory sequences of both genes have now been shown to accurately direct spatial and temporal expression of associated reporter genes, after injection of the appropriate fusion gene constructs into sea urchin eggs. By using this method of gene transfer the *cis* regulatory domain of the CyIIIa gene has been delimited. Coinjection of excess quantities of DNA fragments containing subregions of the regulatory domain results in *in vivo* competition, and thus it is possible to analyse the functional significance of individual regulatory elements. DNA-protein binding studies *in vitro* demonstrate eight sites where highly specific interactions occur within this domain (10^4-10^5 fold preference for CyIIIa site vs. random DNA sequence). Some of the factors that bind to these sites are probably zygotic gene products, since their concentration increases as development proceeds and they cannot be detected in extracts of unfertilized eggs. However, others are clearly maternal, and are stored in unfertilized egg cytoplasm. Analysis of the origin, the cytological distribution and possibly the activation of certain of these CyIIIa regulatory factors in the egg and early embryo, considered in conjunction with the lineage and location of the aboral ectoderm precursors, should, we believe, provide a molecular interpretation of how the CyIIIa gene becomes differentially expressed as the aboral ectoderm is formed. The same factors are likely to regulate other aboral ectoderm genes as well. This is indicated by *in vitro* intergenic competitions, carried out with regulatory regions of other genes expressed specifically in the same cells. An interesting result follows from introduction of the CyIIIa regulatory sequences into eggs of a different sea urchin species. Though the exogenous fusion construct is regulated temporally in a correct manner, spatial regulation is wholly deranged, thus providing an opportunity to identify these *cis*-*trans* interactions required normally for spatial control.

Fig. 1. *In situ* hybridization experiments carried out on 5 μm sections of pluteus stage *Strongylocentrotus purpuratus* embryos. (*a*) and (*b*), (*d*) and (*e*) show sections of 70 hr plutei hybridized with a tritiated antisense probe for the 3' untranslated region of CyIIIa actin mRNA; exposure 2½ months. The embryos shown in (*a*)-(*c*) are sectioned along the oral (upper) - aboral axis; embryo (*c*) has a fold of labeled aboral ectoderm at the upper right lying over its oral surface. Embryos (*d*)-(*e*) are sectioned across the oral-aboral axis, though somewhat obliquely. All of the ectoderm shown is aboral, since the plane of these sections lies below the oral ectoderm. Regions of the gut can be seen internally; and the anus is to the upper left. In *S. purpuratus*, the CyIIIa actin gene is expressed only in the aboral ectoderm. (*a*) and (*d*), phase-contrast; (*b*) and (*e*), darkfield illumination. (*c*) and (*f*), *S. purpuratus* embryos from eggs injected with approximately 2000 molecules of CyIIIa•CAT, photographed under darkfield illumination. The sections were reacted with a tritiated RNA antisense probe for the CAT message, and exposure was for one month. Only cells of the aboral ectoderm are labeled.

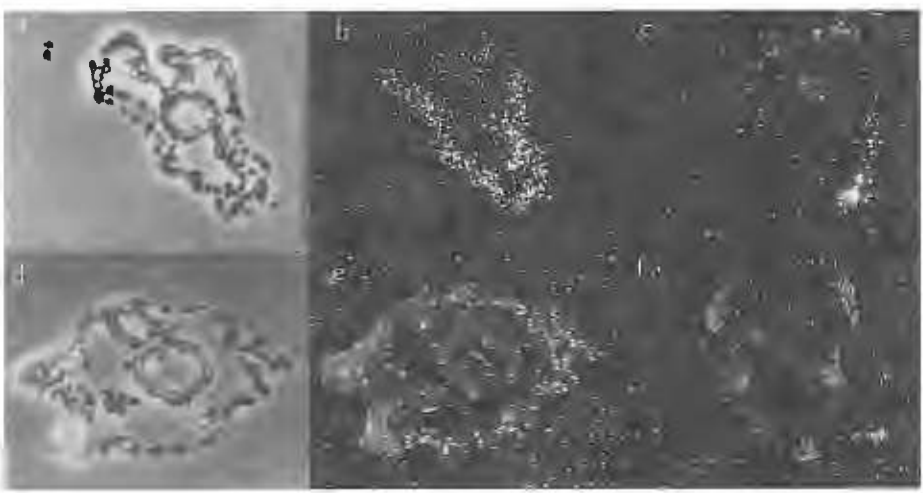
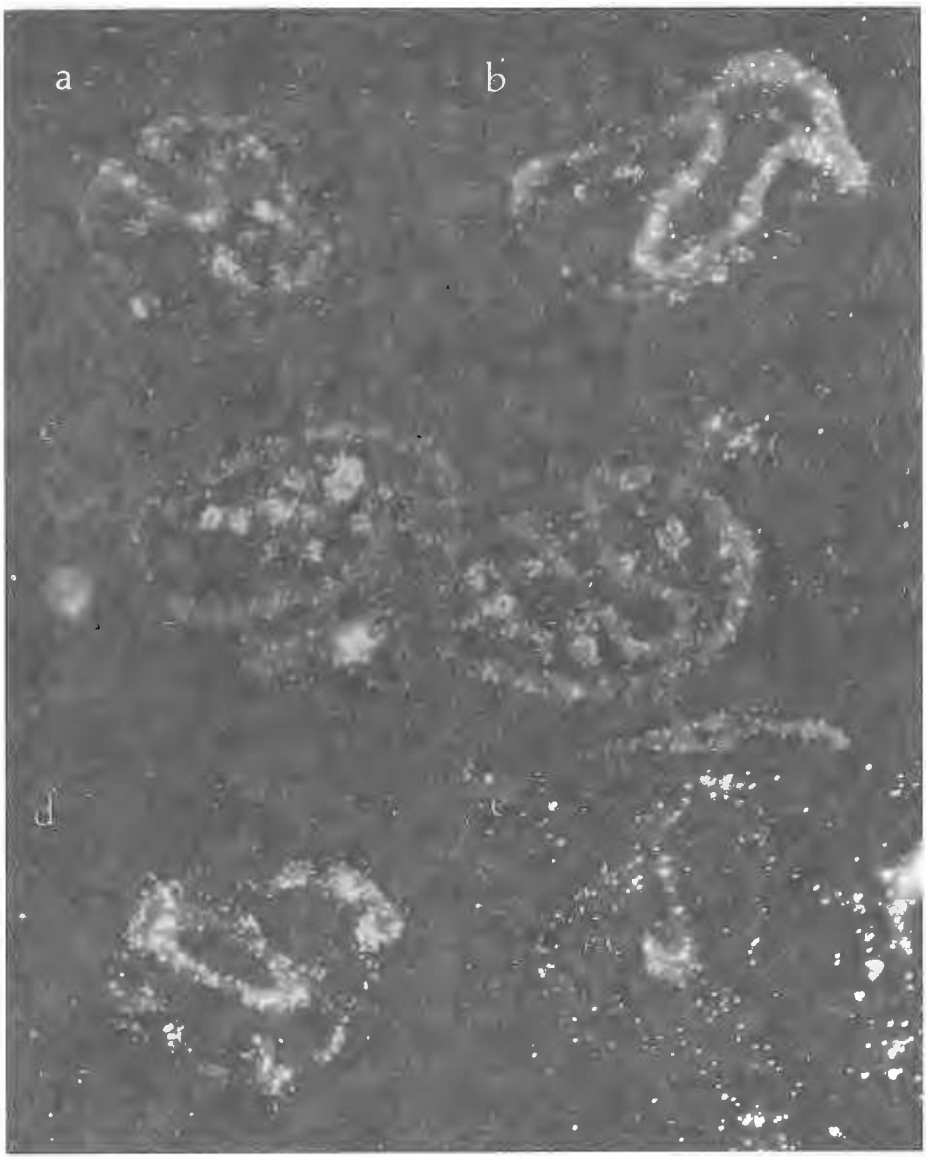


Fig. 2. *In situ* hybridizations of 24 hr *Lytechinus variegatus* plutei from two different series of injections. (*a*)-(*d*), pluteus stage embryos from eggs injected with CyIIIa•CAT. Hybridization conditions were the same as for the *S. purpuratus* CAT hybridizations shown in Figure 3 (*c*) and (*f*). (*a*) Labeling in cells of ectoderm, gut, and primary mesenchyme. The anus is at the upper left in this section, and all of the ectoderm shown is aboral ectoderm. The plane of the section is similar to that of Figure 3 (*d*). (*b*) A few cells of ectoderm, gut, and primary mesenchyme labeled in a second pluteus. In the gut the esophagus is to the lower part of the embryo (orientation was determined by examining serial sections). Note that some of the cells of the oral ectoderm adjacent to the esophagus are labeled, as well as aboral ectoderm cells elsewhere. (*c*) Sections of two plutei labeled primarily in mesenchyme cells. In each embryo the section passes through the anus. (*d*) Heavily labeled embryo, second injection series. The gut is sectioned through the esophagus and stomach, and labeled oral ectoderm appears at the upper left. (*e*) Control *L. variegatus* pluteus (developed from an egg that was not injected) from the same slide as the section in (*d*). Sections of control embryos were included on each slide and carried through the entire *in situ* hybridization procedure along with sections of experimental samples. No cells are labeled. The bright background glow over parts of the tissue in (*d*) and (*e*) is an artifact of the darkfield illumination.



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EMBRYONIC INDUCTION

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Embryonic induction appears to be the single most commonly used mechanism in the development of the vertebrates for bringing about gene activation, cell differentiation, and morphogenesis. Among the invertebrates, the same process seems to be important in the development of Drosophila and nematodes. Though discovered in 1901, the process is not yet understood in any detail at either the cellular or molecular levels.

This contribution will describe new attempts to analyse embryonic induction. The work is being done in amphibia, the material used by Spemann in his classical work and in much subsequent work in this field. One difficulty which has affected previous attempts to analyse embryonic induction over several decades has been the indirect histological assays used to recognise inductive effects. It has been customary to place two tissues in contact, allowing induction to take place, and then to wait for a week or more before analysing the results. Most inductions are complex processes in which two tissues reciprocally induce each other, leading by many sequential steps to a complex end result. We have therefore attempted to simplify the analysis by selecting an inductive process which takes place over a very short time of only a few hours, and by using a molecular marker strongly expressed in the induced cells. The particular induction which will be discussed in this talk is the first one known to occur in amphibian development; this is when vegetal cells of a blastula induce some of the nearby animal cells to become mesoderm and most of these to form muscle. If the animal cells had not received an induction, they would have become epidermis, expressing a cytokeratin gene. There is, therefore, a major switch in the direction of differentiation of blastula animal cells according to whether or not they receive an induction from vegetal cells. As a marker of muscle differentiation, we have used a muscle-specific gene, coding for cardiac actin. Cardiac actin is the major actin component of the skeletal muscle of the early embryo, even though it is expressed in the adult mainly in heart muscle. In the work to be described, we have recognised muscle gene expression biochemically in groups of cells, and by antibodies for individual cells. For biochemical assays, which have the advantage of rapidity and good quantitation, we have used a gene-specific probe coupled with nuclease protection. The probe we have used is complementary to part of the 3' untranslated region of the message and also to part of the coding region. This has the advantage of enabling us to detect all actin transcripts, whether muscle-specific or cytoskeletal, as well as gene-specific mRNAs, using the same probe and same assay. For most experiments we use an RNA probe transcribed from an SP6 promoter. The full length probe of just under 400 bases is protected for 280 bases by the muscle-specific mRNA and for 130 bases by cytoskeletal actin mRNAs.

One of the most important characteristics of embryonic induction is competence or responsiveness. The way in which cells respond to induction is determined very largely by their responsiveness at the particular time of an induction. For example the time at which animal cells which have received a vegetal induction first transcribe their muscle actin genes is related to the age of the animal cells and not to a certain number of hours

after the vegetal contact was made. Similarly the nature of the response which animal cells make to an induction is determined by their responsiveness at a particular stage in development. Thus animal cells at the blastula stage have the choice of being induced, in which case they become mesoderm, or not being induced, in which case they become epidermis, expressing cytokeratin genes. Then at the succeeding gastrula stage, animal cells may be induced by mesoderm tissue to become nerve. They have however lost their competence to respond to vegetal cells and become muscle, by this stage. Thus cells seem to go through a series of competencies or abilities to respond in particular ways. It seems that several different kinds of inducer substances can have the same effect, so long as they act on responding cells at a particular stage in development. Thus the specificity of an induction reaction seems to depend not only on the nature of the inducer substances, but also very importantly on the competence of the responding cells.

There are two levels at which it is useful to analyse any example of embryonic induction, the cellular and the molecular. Two questions need to be answered at the cellular level. One is why only a minority of animal cells competent to respond to vegetal induction in fact do so, and the other is how there is such a sharp demarcation between cells which respond to the induction maximally and those adjacent cells which do not respond at all. Several different experiments have shown that inducing and responding cells do not need to be in direct contact, and that the inducer substances must be diffusible. Several different kinds of experiments have been used to show that different processes contribute to restricting the number of animal cells which respond to the inducing stimulus. These include the number of vegetal cells which contribute inducer, the time at which animal cells lose their competence, and probably also a so-called "community effect" by which animal cells can respond only if influenced by their neighbours responding in the same way. It is worth emphasising the importance of processes which limit the number of cells which respond to induction. In this case, too many cells being induced to form mesoderm would reduce the number of cells able to form nerve, and the whole axis of the embryo would be reduced or eliminated.

The second type of analysis which needs to be carried out on embryonic induction is of a molecular kind, designed to identify the molecules which contribute the inducing and responding effects. We have emphasised above the importance of competence, in addition to the nature of the inducing molecules. Work from several laboratories has recently implicated growth factor-like substances as potential inducers. It now seems likely that these inducer substances bind to receptors on responsive cells and that inducer and receptor will lead to the activation of second messengers, as well as to intracellular events already known to follow the action of growth factors in other systems. The key question in embryonic induction is how these second messenger and other intracellular events lead to the activation of a specific set of genes, the same question being applicable to the action of growth factors in other systems. We have concentrated on attempts to identify the cis-acting sequences associated with genes which respond to induction. We have done this by injecting cloned fragments of DNA into fertilized Xenopus eggs, and observing the expression of the injected genes. Most of the work has been done with a cardiac actin gene

promoter attached to the bacterial CAT gene. We find that only a short sequence of this cardiac actin gene promoter, namely the first few hundred bases, is required to confer inducibility on the bacterial CAT gene. Therefore an induction response element is located in the promoter region of the cardiac actin gene. Trans-acting factors must interact with this region, and several experimental approaches are now being applied in order eventually to identify these factors. We do however know that the induction process which we study absolutely requires the synthesis of proteins in the first part of the reaction. We presume that these proteins will include trans-acting factors which will interact with the cis-acting sequences.

The main emphasis of this talk will be to describe our analysis of a very simple embryonic induction system; we have reduced this system to one in which vegetal cells are exposed to animal cells for three hours and then removed; after this the animal cells need to be cultured for only six hours before the muscle-specific actin genes are transcribed as a result of the induction. The general principles which are emerging from this work may well be applicable to other kinds of short-range cell interactions which take place in development and in adults.

RETROVIRUSES AND EARLY STAGES OF MOUSE EMBRYO

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Certain aspects of early development are difficult to analyze in mammalian embryos. This applies, for instance, to cell lineages or the specificity of gene expression. To study these questions, a strategy has been developed, using recombinant retroviruses.

Retroviral vectors can serve to introduce chosen gene into cells. In multipotential cells, expression does not occur when the gene is under the control of retroviral Moloney virus LTR. Yet, it occurs if another promoter is introduced 5' to the gene. Then transcription starts at the internal promoter in both multipotential and more differentiated cells. Methods have been developed which use the *E.coli*, β-galactosidase Z gene, as a reporter gene to study expression in single cells and to introduce a genetic marker easy to follow in cell lineages.

Two different lacZ genes have been used. One produces an enzyme localized in the cytoplasm of transformed cells. The other produces an enzyme that has been targeted to the nucleus by fusing the Z gene to the location signal of the SV40 large T antigen. Both markers are neutral for cell growth. They do not interfere with *in vitro* differentiation of multipotential cells and can be detected, by histochemical methods, in single cells, even on histological sections with optical or electron microscope. With the nuclear marker, galactosidase appears to be localized at the nuclear periphery in all cells tested, including those of the 2-cell stage embryos.

Stocks of these recombinant retroviruses have been produced and used : 1) to examine lineage of tissues by infecting preimplantation embryos *in vitro* and postimplantation embryos *in vivo* ; and 2) to analyze the properties of different promoters at very early stages of development.

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