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Lineage-specific gene expression and the regulative capacities of the sea urchin embryo: a proposed mechanism

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Summary

Three aspects of early sea urchin development are reviewed, and conclusions derived that lead to a unified concept of how the initial specifications of differential gene activity may occur in this embryo. *i.* The embryo has an invariant cell lineage, and the lineage founder cells can be considered as regulatory spatial domains. That is, from each of these cells descend clones of progeny the members of which express the same set of lineage-specific genes. *ii.* From the extensive classical literature on blastomere plasticity, and some key modern experiments, are derived a system of inductive blastomere interactions, which accounts for the conditionality of lineage founder cell specification. That is, the fates of many of the lineage founder cells can apparently be altered if the normal spatial interrelationships within the embryo are perturbed. *iii.* Recent studies have been carried out by gene transfer, and are supported by *in vitro* analyses of DNA–protein interactions in the regulatory regions of two genes that are expressed in a lineage- specific manner. Expression of both of these markers of cell fate specification is controlled by diffusible DNA-binding factors (i.e. within each nucleus). A molecular mechanism is proposed, based on inductive effects on gene regulatory factors, which in principle provides a specific explanation of the regulative capacities for which this embryo is famous.

Key words: lineage-specific gene expression, regulation, sea urchin embryo, DNA, protein.

Introduction

The sea urchin embryo has served as a major subject of descriptive and experimental investigation for well over a century, and the successive interpretations offered over this long period have reflected faithfully the changing paradigms of developmental biology. Experimental fertilization and embryonic development of sea urchins were first described in 1847 (Derbès, 1847; von Baer, 1847). Pronuclear fusion was discovered in sea urchin eggs some 30 years later (Hertwig, 1876), and the initial observations of egg polarity and cell lineage in the sea urchin embryo date back to Selenka (1883). The important conceptual achievements of the classical experimentalists who followed these embryological pioneers include the cell marking experiments that led to a general, and for certain regions of the early embryo, specific description of cell lineage (Boveri, 1901*a*,*b*; various papers of Hörstadius in the 1930's, reviewed by Hörstadius, 1973, pp. 14–22); the demonstration of Boveri (1902) that a complete genome must be present in all the blastomere nuclei in order for normal development to procede; the demonstration of Loeb (1899, 1909), followed by many others (reviewed by Ishikawa, 1975), that sea urchin eggs are capable of efficient parthenogenic development; and the startling and much repeated observation first made by Driesch in 1891 that any of the meridional blastomeres of 2-cell or 4-cell sea urchin embryos are capable of forming a complete embryo if cultured in isolation (Driesch, 1891, 1900; Boveri, 1907; Hörstadius & Wolsky, 1936; reviewed by Hörstadius, 1973, pp. 92–99). Perhaps the most significant of classical advances, at least for what follows in this essay, was the long and brilliant series of blastomere recombinations carried out by Hörstadius and his associates. These experimental *tours de force* showed that blastomeres of chimeric embryos display developmental fates different from their normal fates, depending on the cellular constitution of the chimeras, and on the ectopic blastomere appositions that had been experimentally created. These studies, many significant examples of which are reviewed in this paper, were interpreted in terms of embryonic gradients, we would say of morphogens, following ideas initially developed by Child (1916*a*,*b*). To accommodate the results of the chimeric embryo experiments Runnström and Hörstadius recast earlier gradient theories in the form of a double gradient emanating from the animal and vegetal poles of the egg (reviewed by Runnström, 1975; Hörstadius, 1939, and 1973, pp. 67–91). Though now standard, and ensconced in most textbooks, this interpretation may be less than satisfactory from the

viewpoint of mechanism, as discussed below. In fact the sea urchin embryo appears to defy any of the doctrinaire interpretations couched in traditional terms. Thus it has an invariant cell lineage, and in the animal–vegetal (A–V) axis there is good evidence for cytoplasmic localization and a preformed egg polarity; but, on the other hand, it is also clearly an inductive system, in which most of the cell fates remain plastic and easily alterable far into cleavage.

Much of our current molecular, cellular, and biochemical knowledge of sea urchin embryogenesis concerns properties that are not *spatially* diversified in the embryo, or that are not immediately concerned with the fundamental early developmental process by which various spatial domains are initially assigned their different fates (*specification*). Examples are the sophisticated analyses now available of the embryo-wide activation of protein synthesis after fertilization; of the expression of histone genes that takes place in all the dividing cells of the embryo; and of the overall utilization of genetic information required for embryogenesis. In the last several years, advances in experimental technology have provided the opportunity to return to the classically defined problem of specification of cell fate, but in ways wholly different from those available to our predecessors. We possess at this point a much improved knowledge of the cell lineage of the sea urchin embryo, and there is an expanding set of recombinant DNA and immunological markers, the expression of which is specific to given cell types and cell lineages. Using such markers gene transfer methods have been applied in order to examine the gene regulatory processes by which arise the diverse cellular structures and functions that begin to be apparent in the sea urchin embryo when it consists of only a few hundred cells. Armed with the early results of these molecular approaches, I attempt in this article a new interpretation of the process by which the sea urchin embryo initially specifies cell fate. To understand this process, it is necessary to integrate our knowledge of the invariant cell lineage of this embryo with the largely older evidence demonstrating the extreme plasticity of most of the blastomeres of which the cleavage stage embryo is composed.

I. Lineage segregation, lineage-specific, differential gene expression in the undisturbed embryo, and conditionality of cell fate

Spatially differential patterns of expression, in which given genes are activated exclusively within given elements of the cell lineage, were noticed in this embryo by Angerer & Davidson (1984). This discovery followed on the development of high resolution, sensitive methods for *in situ* hybridization (Angerer & Angerer, 1981), and the application of these methods to cloned genes that had previously been observed to display sharp temporal changes in expression early in development (Bruskin *et al.* 1982; Shott *et al.* 1984). Lineage-specific spatial patterns of gene expression (in the unperturbed embryo) are the consequence of an invariant pattern of cleavage, in which clones of given embryonic cell types and structure always derive from the same founder cells, since the specific functional characters of these cell types ultimately requires that particular genes be expressed in them. The implication is that the founder cells of such lineages constitute spatial domains of egg cytoplasm within which specific sets of genes will be active. The significance of this phenomenon is obvious, for the mechanism by which it occurs would explain the initial spatial variation in gene expression seen in the sea urchin embryo, from which follows subsequent developmental events, and the additional diversification of gene expression that they in turn require.

Present knowledge of the cell lineage of this embryo, and the current catalogue of lineage-specific molecular markers have been recently reviewed elsewhere (Davidson, 1986, pp. 213–246, 494–504; Cameron *et al.* 1987). This is now an active area of research, and new information is becoming available both with respect to yet unresolved areas of the lineage itself, and to spatial patterns of gene expression. The major conclusions, as they now appear, are described in the following two sections.

1. *Founder cells, lineage elements, and the structures of the embryo*

Five embryonic *territories* can be distinguished by the blastula stage, each defined as a contiguous region of the single-cell-thick, spherical blastula wall. These territories are distinguished by specific, differential patterns of gene expression (or the absence of particular gene activities), as discussed below; by their cell lineage; and by the nature of this progeny. The embryonic cell fate of each territory is unique. These territories are: the prospective aboral ectoderm, the prospective oral ectoderm, the prospective skeletogenic mesenchyme, the vegetal plate, and the eight 'small micromeres.' Each of these territories derives from a specific set of founder cells that segregate at 3rd–6th cleavage. A *founder cell* is here the first cell to arise in each region of the lineage, all of whose progeny contribute to only one territory. From embryo to embryo given founder cells occupy exactly the same position in the lineage map, and are found in approximately the same locations with respect to the axes, in the three-dimensional cleavage-stage embryo. There is no cell migration until midblastula stage, when skeletogenic mesenchyme cells enter the blastocoel. The territories of the early blastula are each, then, composed exclusively of the clones generated from its several founder cells, while each such clone contributes exclusively to one contiguous territory. In Fig. 1 is shown a colour-coded lineage diagram through 6th cleavage for regularly developing sea urchins (there are 9 or 10 cleavage divisions in all, depending on the species). The set of clonal founder cells giving rise to each of the territories is indicated by the solid circles in the lineage map of Fig. 1, and the approximate locations of the different nascent territories are indicated

by the coloured regions superimposed on a photograph of a living ~6th cleavage embryo (top right).

The relation between the initial territories and the structures and cell types of the gastrula and pluteus stage embryos differs according to the example chosen. A set of molecular functions has been identified that is expressed by all the cells of the eleven clones constituting the *aboral ectoderm* territory. The prospective aboral ectoderm clones thus appear to give rise to a single differentiated cell type, and produce a single embryonic structure [it should be noted that at the pluteus stage expression of a homeobox gene initially transcribed throughout the aboral ectoderm becomes confined to the progeny of the VA cell (Dolecki *et al.* 1986); in addition, late in postembryonic larval development several patches of the larval aboral ectoderm redifferentiate to form adult dorsal epidermis (Cameron *et al.* 1989)]. The fate of the prospective *skeletogenic mesenchyme* territory is similarly straightforward. The four clones constituting this territory undergo three cleavages following the 5th cleavage segregation of their founder cells, all of the progeny of which ingress into the blastocoel and embark on a sequence of molecular and morphological differentiation which culminates in skeleton formation. A further multiplication of these cells occurs late in embryogenesis. These skeletogenic lineage elements thus also produce only a single differentiated cell type that forms a single embryonic structure and expresses a unique set of genes. Late in embryonic development the functions of individual skeletogenic cells must vary depending on where they are in respect to the growing skeletal rods. In any case the one-to-one relations that obtain between prospective aboral ectoderm and prospective skeletogenic mesenchyme territories and the embryonic structures they ultimately produce are the least complex in the embryo. It is these lineage elements with which we are mainly concerned in the following.

The products of the other territories are more diverse. The *oral ectoderm* ultimately produces a morphologically involved set of epithelial facial folds, the oral hood, the mouth, the ciliated bands, and most or all of the larval nervous system. The *vegetal plate* remains an indifferent single-cell-thick epithelial disc until the end of the blastula stage, but then gives rise to many different late embryonic cell types and internal larval structures, including a variety of mesenchymal derivatives, gut, and coeloms. It is not known whether there are early lineage decisions *within* the vegetal plate, perhaps dating back to cleavage, or if on the other hand these cells remain pluripotential irrespective of their positions or their specific ancestries within the vegetal plate. In both oral ectoderm and vegetal plate territories a late developmental diversification thus occurs, though the pattern of molecular activities expressed by the cells deriving from these territories reveals a long lasting imprint of their initial lineage affiliations, as noted in the following section. Little is known of the eight 'small micromeres' which remain as such throughout embryogenesis, except that they may ultimately contribute to the coelomic rudiments (Pehrson & Cohen, 1986).

2. *Lineage-specific gene expression in the skeletogenic mesenchyme and the aboral ectoderm*

Batteries of genes and gene products that characterize the differentiated cells of the aboral ectoderm, and of the skeletogenic mesenchyme, are now being identified (for nomenclature, see Table 1). For example, 9 or 10 proteins constituting the skeletal (spicule) matrix and synthesized by skeletogenic mesenchyme cells were characterized by Benson *et al.* (1987), and a number of other proteins that are synthesized specifically and exclusively by these cells have been detected by immunocytological methods and by cDNA cloning (reviewed by Davidson, 1986, pp. 224–228; Harkey *et al.* 1988). Similarly, genes for two cytoskeletal actins, and for a family (in *S. purpuratus*) of about eight Spec 1 and 2 proteins, related to the calmodulin/troponin C/myosin light chain group of calcium-binding proteins are expressed specifically in aboral ectoderm (reviewed by Davidson, 1986, pp. 229–233; Hardin *et al.* 1988). The pattern of known gene expression in these lineages is of course complex, and includes the following elements. (a) Genes that are activated very early, i.e. soon after lineage founder cell segregation is complete: the cardinal cloned examples are for aboral ectoderm, the genes coding for the CyIIIa cytoskeletal actin and the Spec 1 protein, and for the skeletogenic mesenchyme, the

Table 1. *Specifically expressed genes referred to in text*

Gene	Product or function	Where expressed
Spec 1 Spec 2	Members of calmodulin/troponin C/myosin LC family	Aboral ectoderm
CyIIIa	Cytoskeletal actin	
SM50	50×10^3 M_r spicule matrix protein	Skeletogenic mesenchyme
msp130	Protein required for migration or Ca^{2+} uptake	
CyIIa	Cytoskeletal actin	Vegetal plate derivatives and skeletogenic mesenchyme
CyI CyIIb	Cytoskeletal actins	Ubiquitous early; Oral ectoderm and gut late
Spec 3	Ciliary structural protein	Oral and aboral ectoderm

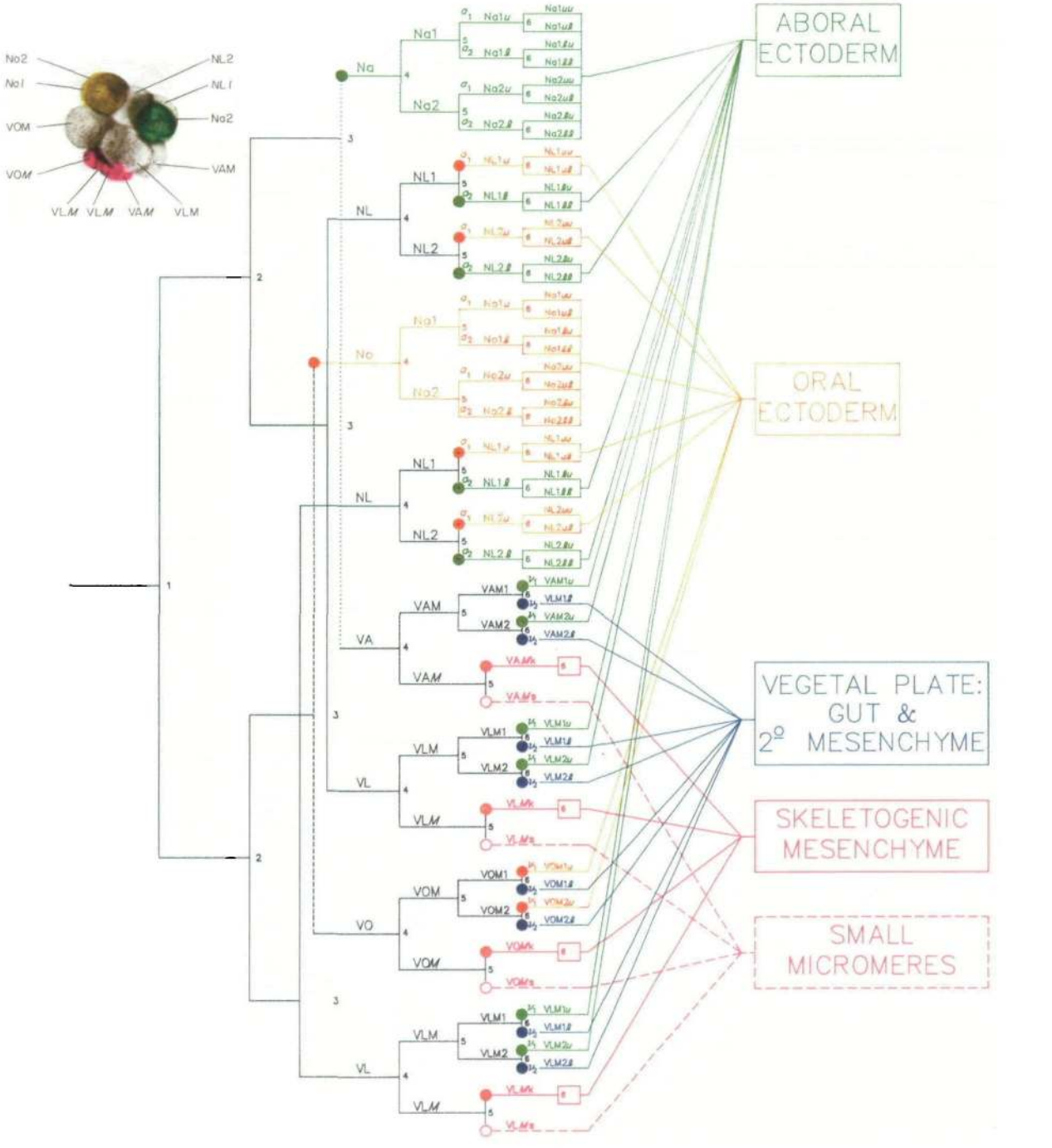
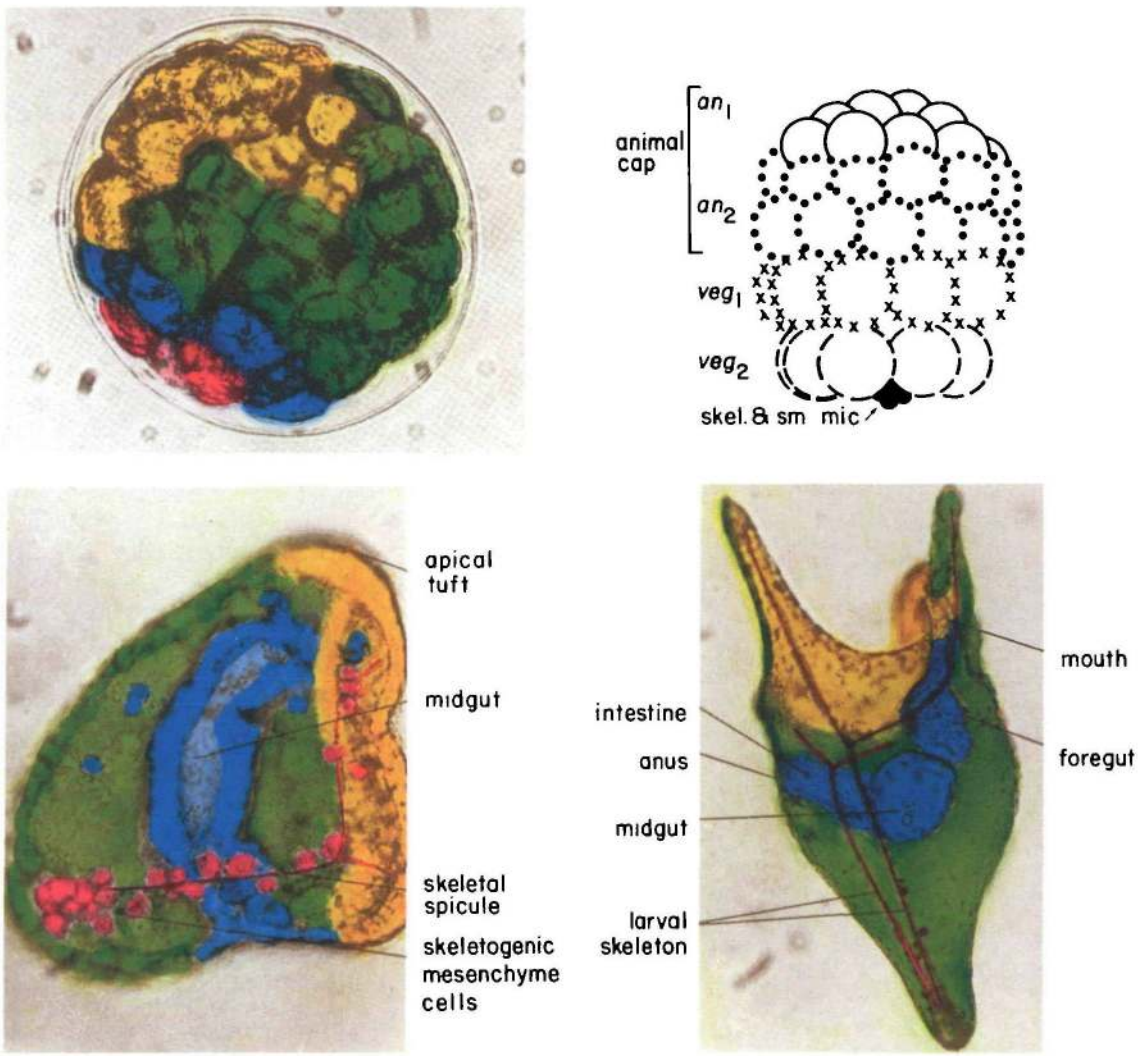


Fig. 1. Lineage of regularly developing sea urchin embryo to 6th cleavage, and location in living blastula, gastrula, and pluteus of various lineage elements. A lineage diagram is shown on the left. For sources, references, and details see Fig. 4.5 of Davidson (1986), and Cameron *et al.* (1987); unpublished results of Cameron and the author are drawn upon as well in reference to the exact clonal origins of the VL aboral ectoderm lineage. Nomenclature: N, animal; V, vegetal; L, lateral; O, oral; A, aboral; *M*, 4th cleavage micromeres and their descendants; M, the 4th cleavage sister cells of the micromeres ("macromeres") and their descendants; k, skeletogenic lineage; s, small micromere lineages. The animal half of the embryo is shown at the top and the vegetal half at the bottom of the lineage map. The NL and VL blastomeres of the lateral quadrants are connected by solid lines; the oral blastomeres No and VO are similarly connected by a dashed line; and the aboral blastomeres Na and VA are connected by a dotted line. The products of horizontal cleavage planes after 3rd cleavage are denoted lower (*l*) and upper (*u*) (with respect to the animal–vegetal axis); the products of the more or less vertical, radial cleavages as (1) and (2). The successive cleavages are denoted by the small Arabic numerals at the branch points of the diagram. The convention used in this diagram is that cells whose progeny contribute to more than one of the *territories* (see text) indicated in the boxes to the right of the lineage map are denoted by capital letters. In the names of *clonal*



founder cells, all of whose progeny contribute to only one territory, the last symbol is lower case. These founder cells are denoted by the solid, respectively coloured circles. v_1, v_2, a_1, a_2 denote the veg_1, veg_2, an_1, an_2 tiers of Hörstadius (see text), as indicated in his tier diagram, which is reproduced at the upper right. The positions of the vertical branch points at 4th–6th cleavage in the lineage map indicate temporal relations of the various cleavages, which are somewhat delayed in the micromere lineages; these relations vary in different species (and temperatures), and the lineage shown in the animal quadrant has only been examined carefully to our knowledge in *S. purpuratus*. The lineages leading to each territory, and the parts of the embryo descendant from each in the lineage map, and throughout Fig. 1, are colour coded: *green*, aboral ectoderm; *yellow*, oral ectoderm; *blue*, vegetal plate, giving rise to gut and secondary mesenchyme; *red*, skeletogenic mesenchyme; *pink*, small micromeres. At the upper left of the map is a photograph showing a 16-cell embryo, in which oral and aboral polarity are already indicated (Czihak, 1963). The three-dimensional positions of the visible cells is indicated; cf. based on lineage map (Davidson, 1986). In the right-hand portion of the figure are early blastula (about 6th cleavage), gastrula and pluteus stage embryos of *Paracentrotus lividus*. Embryos of this species are unusually transparent (photograph of 6th cleavage embryo from Dr Giovanni Giudice, reproduced with his kind permission). In this early blastula the embryo has *arbitrarily* been considered to be oriented so that a lateral aspect faces the observer; oral left, aboral right. The animal–vegetal axis is obvious from the position of the polar small micromeres. The leftward protruding patch of aboral ectoderm cells in the mid tiers of the embryo are progeny of the *lower* 5th cleavage NL offspring and of VL blastomeres (see lineage map). The upper sisters of these NL descendants produce the ciliary band of the oral ectoderm. The small micromeres, now located at the tip of the archenteron (Pehrson & Cohen, 1986) are not shown in the gastrula, or in the pluteus, where they contribute to the coelomic sacs, which are not clearly visible in this photograph. Many more skeletogenic and secondary mesenchyme cells are present in the gastrula stage embryo than are clearly in the plane of focus. In the pluteus, except for the surface of the more distant oral arm, the oral ectoderm is seen *through* the external aboral ectoderm and is hence coloured yellow-green. The oral hood which rises at the back of the mouth is pointing upward on the right side as shown, and is perpendicular to the plane of this picture.

SM50 gene, which codes for a 50×10^3 M_r spicule matrix protein. (b) Genes that are ever thereafter *precluded* from activation, in that lineage: an example is the CyIIa cytoskeletal actin gene, which is expressed relatively late in development, and then only in vegetal plate and mesenchyme lineages, and never in aboral ectoderm (Cox *et al.* 1986). (c) Genes that are initially active everywhere, but expression of which is gradually confined: examples are CyI and CyIIb cytoskeletal actin genes, which are initially globally expressed but ultimately are silenced in aboral ectoderm while continuing to be actively expressed in oral ectoderm and gut (Cox *et al.* 1986). (d) Genes that are activated relatively later in the development of their lineages: Examples are the gene coding for a cell surface mesenchyme cell glycoprotein known as msp130 which may be required for mesenchyme cell migration or for Ca^{2+} uptake, the product of which is detected only after ingression of these cells into the blastocoel (McClay *et al.* 1983; Grant *et al.* 1985; Wessel & McClay, 1985; Anstrom *et al.* 1987; Leaf *et al.* 1987); or for another recently cloned spicule matrix protein gene that begins to be expressed in skeletogenic mesenchyme some hours after the SM50 gene does (F. Wilt, pers. commun.); or the several Spec 2 genes that have been characterized by Hardin *et al.* (1988) and that begin to be expressed in aboral ectoderm only at the late blastula–gastrula stage, many hours after Spec 1 expression is initiated in the same lineages. While all of these patterns of expression are indeed interesting we focus here on the first class, as it is genes such as CyIIIa, Spec 1 or SM50 that serve as markers of the earliest known lineage-specific gene expressions, and that might therefore be considered to be immediately controlled by the process of lineage specification *per se*. If lineage founder cells define domains of future differential gene expression, the regulatory molecular biology of these genes might provide a direct pathway into the key mechanism by which specification affects gene expression.

Measurements of CyIIIa and Spec 1 transcript accumulation (Lynn *et al.* 1983; Shott *et al.* 1984; Lee *et al.* 1986; Nemer, 1986) and nuclear run-off studies (Lee, 1986; Hickey *et al.* 1987) show that these genes are abruptly activated late in cleavage. Prior to this they are silent, though overall, transcriptional rates are higher in cleavage-stage sea urchin embryo nuclei than ever thereafter (reviewed by Davidson, 1986, pp. 174–180, 140–160). The egg and early cleavage embryo contain a relatively small amount of Spec 1 and CyIIIa maternal mRNAs, i.e. 1–2% of the ultimate level of accumulation of each message. At the earliest stages at which CyIIIa and Spec 1 expression can be detected, the newly synthesized transcripts are confined to the cells of the prospective aboral ectoderm (Cox *et al.* 1986; Hardin *et al.* 1988). Activation of the CyIIIa gene probably occurs in these cells at 7th–8th cleavage, if not earlier, i.e. only one or two cleavage divisions past the last divisions required for clonal segregation of all of the aboral ectoderm founder cells. Though as yet less intensively studied, similar conclusions obtain for the SM50 gene. This gene is also silent early in cleavage, and from the time the SM50 gene is first activated it is expressed exclusively in cells of the prospective skeletogenic mesenchyme lineage (Benson *et al.* 1987; Sucov *et al.* 1988; Killian & Wilt, 1989). It too appears to be transcriptionally activated late in cleavage, perhaps shortly after the delayed 6th or 7th cleavage divisions characteristic of the skeletogenic mesenchyme lineage (see Fig. 1) i.e. again only one or two divisions following the final segregation of the lineage founder cells.

For other territories that give rise to more diverse arrays of cell types and structures, early lineage segregations are nonetheless still significant in terms of regulation of gene expression. The example of CyIIa actin expression has already been noted, a case in which the later domain of expression (gut plus skeletogenic and secondary mesenchyme) is actually the sum of two of the primary territories shown in Fig. 1, *viz.* the vegetal plate and skeletogenic mesenchyme territories. Spec 3, an ectoderm-specific ciliary structural protein, is expressed in the sum of the oral and aboral ectodermal territories, but not, e.g. in the gut, which derives from the vegetal plate territory, even though a portion of the gut also has cilia (Eldon *et al.* 1987). In addition certain antigens detected by monoclonal antibodies are expressed in various derivatives of the vegetal plate territory, even though these form diverse cell types and structures, e.g. an endodermal antigen, which is expressed prior to archenteron invagination throughout the vegetal plate, and later in the (functionally distinct) cells of intestine and stomach (Wessel & McClay, 1985). Thus, even where there is not a one-to-one relation between lineage territory and subsequent cell type, the initial regions of the egg cytoplasm that are divided up amongst the various founder cells constitute domains in which specific patterns of gene expression will subsequently be instituted. In these cases the sharing of a given gene expression among histologically distinct cell types, which also share a primordial origin in the lineage map, can be regarded as a profound indication of the mechanism by which particular patterns of gene activity are initially specified.

3. *Invariant cell lineage but conditional cell fate: a reinterpretation of classical evidence*

Though the early cleavage pattern leading to the segregation of the various lineage founder cells in regularly developing sea urchin embryos is invariant, its *functional significance* is clearly different from that implied by the classical theory of mosaic development. Classically, lineage elements leading to diverse structures in embryos displaying 'determinate cleavage' were regarded as spatial domains into which are segregated localized morphogenetic factors, and the invariant cleavage pattern as the devices by which these factors are segregated to the appropriate founder cells. By virtue of their regional inheritance the various descendant lineage elements would thus be enabled, or determined, to differentiate autonomously. Though many kinds of embryo in fact display invariant cell lineage, and some indeed include autonomously differentiating lineage elements descendant from specific

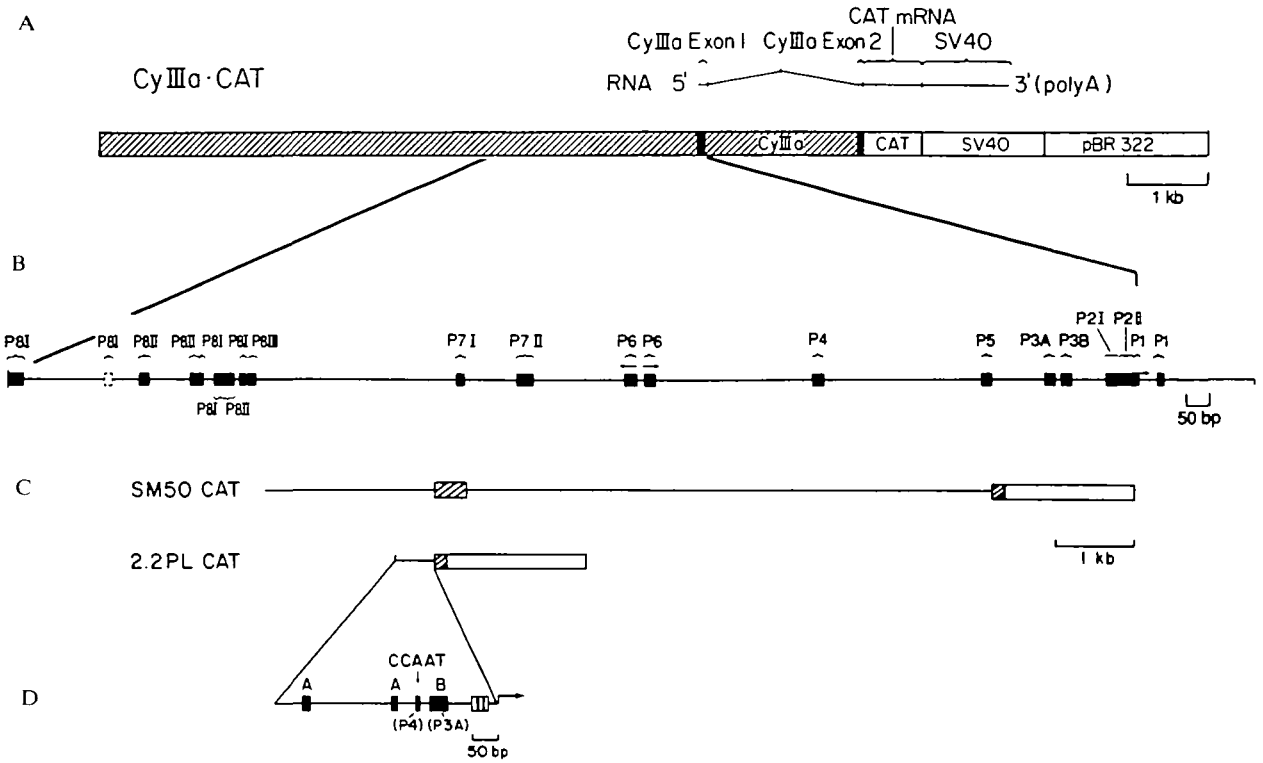


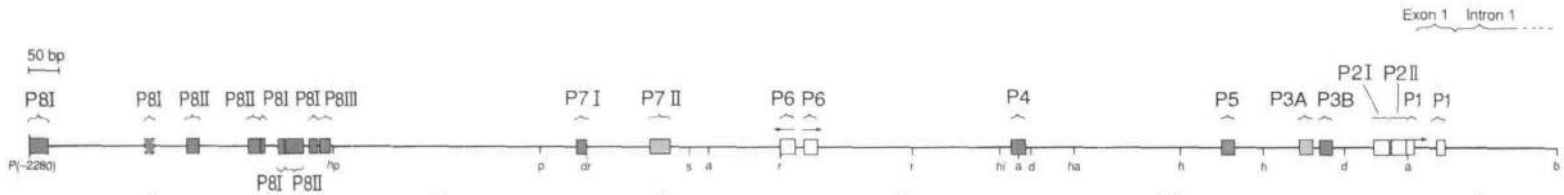
Fig. 2. CyIIIa and SM50 fusion constructs, and location of sites of high specificity interaction with DNA-binding proteins. (A) CyIIIa·CAT fusion gene (Flytzanis *et al.* 1987). The structure of the CyIIIa·CAT transcript is shown above. This begins with a 75 nt leader exon, traverses a ~2 kb intron, and continues into exon II, which contains 25 nt of leader sequence. The fusion site with the CAT gene assemblage is 11 codons into the CyIIIa cytoskeletal actin protein coding sequence. In the diagram of the fusion gene those regions of the sequence deriving from the CyIIIa gene are hatched, and CAT SV40 and vector components are shown as open boxes. CyIIIa·CAT contains about 7 kb of upstream sequence plus the leader intron. This intron does not include sufficient regulatory sequence to permit transcription in the absence of sequence upstream of the promoter (Flytzanis *et al.* 1987), though it could contain yet unknown ancillary sites. (B) The upstream regulatory domain of the CyIIIa gene. The transcription start site is indicated by the small arrow pointing rightward in the P1 binding region. Black boxes denote specific short sequences that have been mapped to within a few nucleotides, where 24 h embryo nuclear proteins are found to bind with high specificity to the CyIIIa DNA (see text). The nomenclature used in text to refer to these various proteins is indicated; each name, e.g. P8I, P8II, P7I, etc., represents a factor binding to a site recognized within the CyIIIa regulatory domain (so far as is now known) only by that factor. P4 is a CCAAT binding factor that also interacts with a CCAAT site in the SM50 gene (see below). The P3A factor interacts with the SM50 gene as well, at the B site. The dotted box in the P8 region represents a putative binding site for the P8I factor for which direct evidence is yet missing. (C) SM50·CAT and the derivative 2.2PL·CAT fusion genes (Sucov *et al.* 1988). Hatched boxes represent exons of the SM50 gene; solid lines other regions of SM50 sequence; open boxes the CAT gene and ancillary assemblage as in C. SM50·CAT includes a ~7 kb intron located in the 35th codon of the protein coding sequence. The fusion to the CAT gene is at codon 43 (see Sucov *et al.* 1988 for details). In 2.2PL·CAT the 7 kb intron is deleted and the fusion to the CAT gene sequence occurs in the 4th codon of the SM50 gene sequence. Only 440 nt of upstream sequence, rather than the 2·2 kb included in SM50·CAT are present, all that is required for active and accurate lineage-specific expression (Sucov *et al.* 1988; see text). (D) The 440 nt minimal regulatory domain of 2.2PL·CAT, symbolism and scale as in B. The striped box represents a complex site of interactions not yet fully analysed.

regions of the egg, no embryo known functions as if it is composed entirely or even largely of autonomous lineages behaving according to this extreme classical model (see review in Davidson, 1986, Ch. 6). An invariant cell lineage means only that in the *undisturbed embryo* the same founder cells always give rise to exactly the same parts of the embryo. An invariant cleavage pattern does not *per se* imply the means by which cell fate is specified, except to say that there could be some relation between the positions of (some) cleavage planes and the process of specification. Numerous experiments, classical and modern, demonstrate the plasticity, rather than the autonomy, of cell differentiation and regional fate in the sea urchin embryo. An interpretation developed in the following is that the cleavage planes themselves function as sites of particular inductive interactions, through which are specified the founder cell fates in most regions of this embryo.

Certain aspects of the spatial organization of the sea urchin embryo depend on the preformed (i.e. maternal) organization of the egg. The egg is primordially polarized in the animal–vegetal (A–V) axis. As first recognized clearly by Boveri (1901*a*), the animal pole is

Fig. 4. Current working hypothesis for biological roles of high specificity sites of DNA-protein interaction within the CyIIIa regulatory domain. A map of the sites is shown at the top, reproduced from Fig. 2B, but colour coded according to the functions shown in the first column of the tabular presentation below. Column 2 lists the factors whose binding sites are shown on the map. Columns 3–6 summarize data on which the factor classifications of Column 1 are based. In Column 3 are symbolized measurements of Calzone *et al.* (1988) which demonstrate the relative factor prevalences in the nuclei of 7 h embryos (left box), when the CyIIIa gene (or CyIIIa·CAT) is silent, *vs.* prevalence of the same factor in nuclei of 24 h embryos, when the CyIIIa gene (or CyIIIa·CAT) is active. Boxes of equal height indicate that the prevalence is the same within a factor of 3 at the two stages; a low 7 h box compared to the 24 h box indicates that factor prevalence increases 10- to 50-fold over this interval. Column 4 indicates the result of *in vivo* competition experiments such as shown in Fig. 3 (Franks *et al.* 1988*c*). '+' indicates competition, and thus that the interaction has a positive regulatory function (e.g. Fig: 3C,D); '0' indicates no competition (e.g. Fig. 3E). It is inferred that factor P3B functions positively although this specific competition experiment has not yet been done, since a fragment bearing both P3A and P3B sites competes effectively, while P3A site alone does not compete, as shown in Fig. 3E. Similarly it is inferred that the P7I site competes though it has not yet been studied separately, because a fragment containing P7I and P7II sites competes, though the P7II site alone does not. Column 5 shows the results of *in situ* hybridization assays of the *in vivo* competitions (B. R. Hough-Evans *et al.* unpublished). These data are still preliminary. Where CAT mRNA appears only in ectoderm, and where aboral ectoderm can be distinguished from oral ectoderm, only in aboral ectoderm, expression is described as 'normal.' 'ND', experiment not yet done; 'ectopic', expression in mesenchyme and/or gut cells as well. Ectopic expression caused by excess binding site indicates an interaction that probably has a negative spatial regulatory function. Column 6 shows the results of intergenic competition studies *in vitro* (P. Thiebaud *et al.* unpublished). Competitions were carried out by gel shift using the regulatory regions, or fragments thereof, of the Spec 1, SM50, and CyI genes (see text). 'None' indicates that none of these particular regulatory DNAs competed with the CyIIIa probe containing the respective binding site; otherwise competition was obtained with regions of those genes noted. Only results indicating that the competing regulatory sequences have an affinity for the respective factor that exceeds 0·5 of that for the CyIIIa site are indicated; in many of these cases the competing sequence in fact displayed a greater affinity (2–10×) for the factor than does the CyIIIa site. The putative 'general enhancer' sites (green), *viz.* P3B, P4, and the several P8 sites, are regions where the interactions that occur *in vivo* act positively, according to competition experiments, (Column 4); and that may be utilized in several differently expressed genes (Column 6). These sites are perhaps less likely to be involved in temporal regulation, because the factors that react specifically with them are present at more or less final concentration in the embryo nuclei well before the CyIIIa gene is activated (Column 3; of course even these factors *could* serve as temporal activators if they were altered so as to become transcriptionally active after 7 h, without affecting either their DNA-binding properties or the migration of their gel shift complexes). Additional relevant evidence is that deletion of the P8 binding region decreases activity of injected CyIIIa·CAT >95 % ($\Delta\downarrow$; Flytzanis *et al.* 1987); and that P4 is a CCAAT site, while P3B is an 'octamer' site (Thézé *et al.* unpublished). The putative 'temporal regulators' (red), *viz.* the P5 and P7I sites, are regions where the interactions act positively (Column 4); but where the factors binding to these sites increase strongly in concentration over the time interval in which CyIIIa gene activation occurs (Column 3). The P5 site is apparently used by all of the three other genes tested (Column 6). Deletion of this site eliminates >85 % of CyIIIa·CAT activity ($\Delta\downarrow$; Column 4; Flytzanis *et al.* 1987). P3a and P7II are possible negative control sites (yellow). These sites alone cause ectopic expression in competition experiments (Column 5), and it is significant that in both cases no competition is observed *in vivo* (Column 4), while *in vitro* both sites may also be involved with regulation of Spec 1 (Column 6). The intergenic competitions (Column 6) also suggest that P1 and P2 could be involved in spatial regulation, since competitive protein binding sites are found exclusively in the Spec 1 control region, among the three other genes tested. If so, P1 and P2 must function positively, according to the *in vivo* competition data (Column 4). Paradoxically, we have no indication of the *in vivo* function of the P6 site, which displays altogether the highest affinity DNA–protein interaction according to the measurements of Calzone *et al.* (1988).

marked by a minute canal in the egg jelly coat that is present prior to fertilization and, in a few species, the vegetal region can be identified by a horizontal (with respect to the A–V axis) ring of pigment granules (Boveri, 1901*a*; Schroeder, 1980; Sardet & Chang, 1985). The polarity of the A–V axis is predictive with respect to cell fate since, as shown in Fig. 1, it is from the vegetal end that skeletogenic mesenchyme and gut normally arise. A second major aspect of maternal spatial organization encoded in the egg cytoplasm is displayed in the (unknown) mechanism that orients the rigidly alternating succession of cleavage planes in different regions of the egg, with respect to the A–V axis. Thus the first two planes are orthogonal and vertical, intersecting along the A–V axis; the third is horizontal and more or less equatorial; the fourth is horizontal and asymmetrically displaced toward the vegetal pole in the vegetal hemisphere, while vertical and radial in the animal hemisphere; the fifth is more or less horizontal in the animal hemisphere but radial and vertical in the vegetal hemisphere; the sixth is horizontal in the vegetal hemisphere but radial in the animal hemisphere, etc. (see Fig. 1). By sixth cleavage, as we have seen, all the founder cells of the territories indicated in Fig. 1 have segregated. Details aside, the important conclusion is that the fixed cleavage geometry determines the position of the founder cells with respect to the preformed A–V axis, and with respect to one another. The localization of the successive cleavage planes is a function of the cytoarchitecture of the egg, and it does not *per se* depend on zygotic genome function. This is shown, for example, by studies of



PUTATIVE FUNCTION	FACTOR	7/24 Hr PREVALENCE	COMP *IN VIVO*	SPATIAL EXPR *IN VIVO* COMP	INTERGENIC COMP *IN VITRO*
GENERAL ENHANCER	P3B		(+)	ND	NONE
	P4		+	NORMAL	SM50, CyI
	P8		+ Δ↓	NORMAL	NONE
TEMPORAL REGULATOR	P5		+ Δ↓	NORMAL	Spec I, SM50, CyI
	P7I		+	NORMAL	NONE
NEG SPACE REGULATOR	P3A		0	(ECTOPIC)	Spec I, SM50
	P7II		0	(ECTOPIC)	Spec I ONLY
?	P2		+	ND	Spec I ONLY
	P1		+	ND	Spec I ONLY
	P6		0	NORMAL	NONE

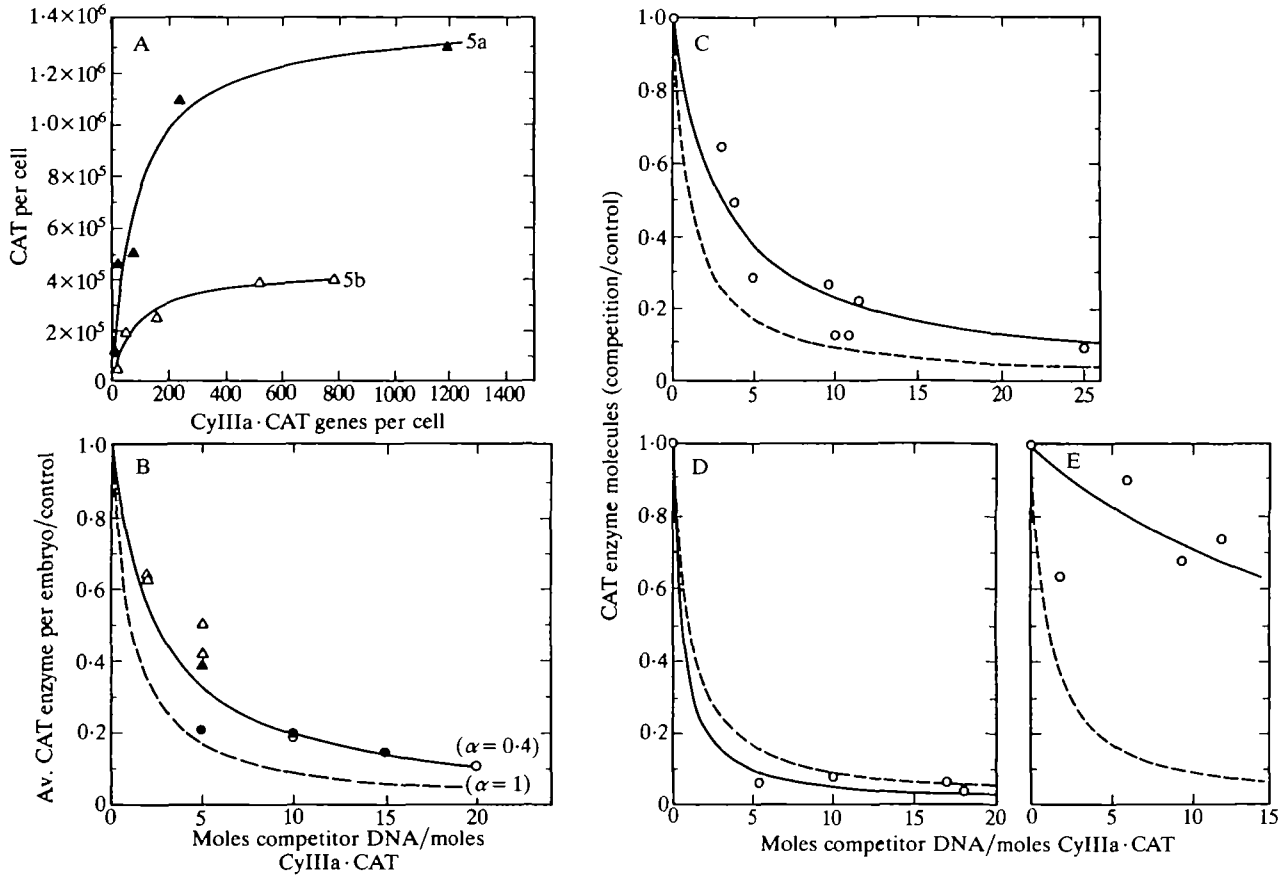


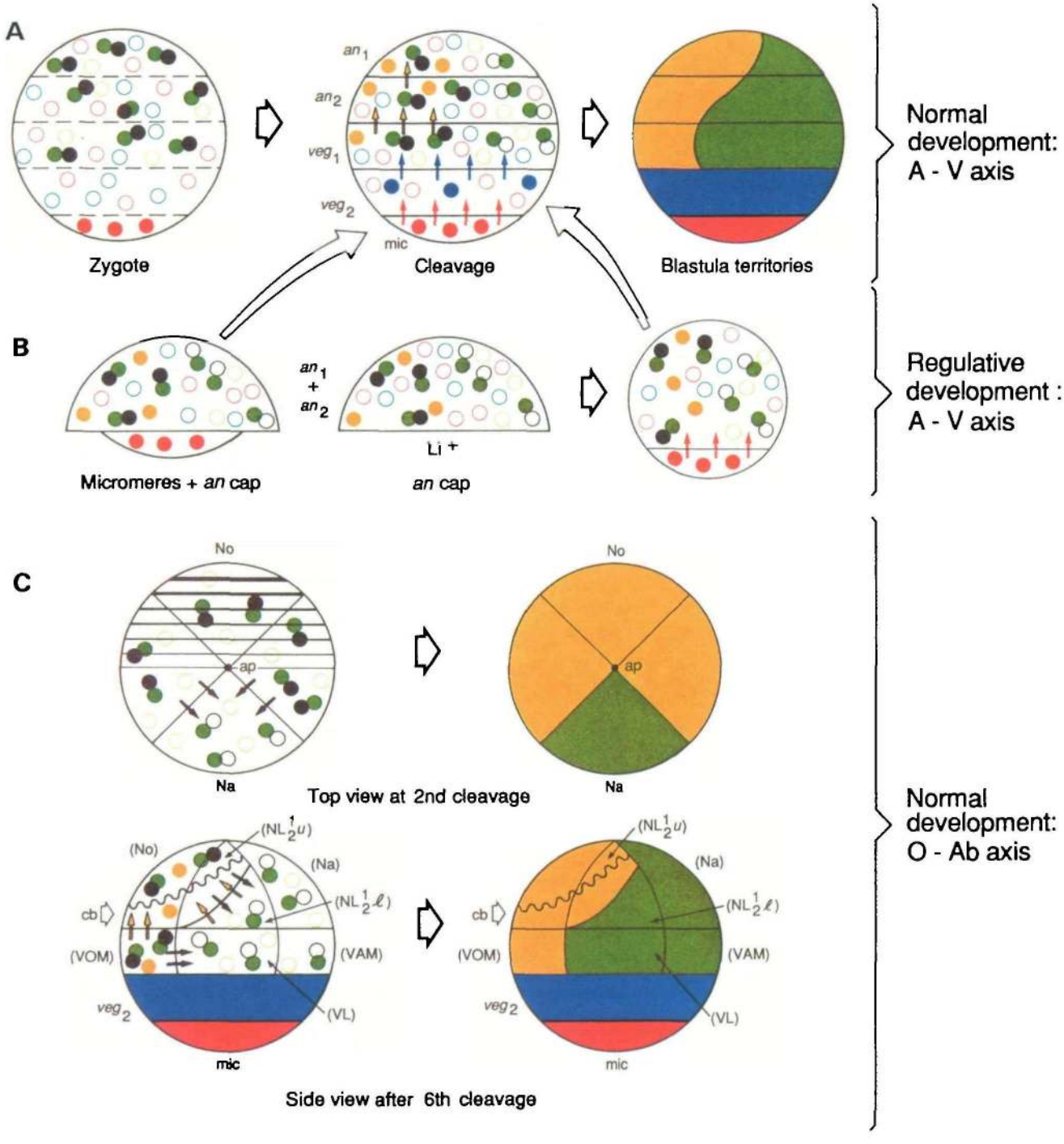
Fig. 3. Competition *in vivo* for factors binding in the CyIIIa regulatory domain. (A) CyIIIa·CAT expression (ordinate) as a function of average number of CyIIIa·CAT genes (abscissa) per cell. Saturation is a requirement for stoichiometric competition, since at saturation the individual regulatory domains compete with one another for limiting factors. Two experiments (5A, 5B) carried out with the two most different batches of eggs are shown. The average of many such experiments indicated 90% saturation at ~1050 genes per cell (average). (B) Near-stoichiometric competition *in vivo*. In the particular experiment reproduced the whole of the regulatory domain shown in Fig. 2B was coinjected with CyIIIa·CAT, in the molar ratios to CyIIIa·CAT indicated on the abscissa. The dashed line shows the ideal competitive stoichiometry; the experimental points are fit by the solid line which is the competition function obtained if the regulatory DNA competes with about 40% of ideal efficiency, or if about 40% of the injected competing sequences end up incorporated in the same nuclei with the CyIIIa·CAT sequences ($\alpha = 0{\cdot}4$). (A–B) From Livant *et al.* (1988) *Proc. natn. Acad. Sci. USA.* **85**, 7607–7611. Data from many experiments (Franks *et al.* 1989) indicate that the mean value of the parameter α for competition with the whole regulatory domain is about 0·75. (C–E). Similar competition experiments carried out with *subfragments* of the CyIIIa regulatory domain; presentation as in B. Data are from Franks *et al.* (1989) *Ciba Symp.*, in press. Excess fragments (ligated into long carrier DNA fragments before injection) containing the binding site for factors P5 (C) and P4 (D) (see Fig. 2B) compete about as strongly as does the whole regulatory domain shown in B. Panel (E) shows that the binding site for the P3A factor does not compete significantly in this assay.

embryos derived from species hybrids (cf. Davidson, 1976, Ch. 2). In this connection the effects of weak detergents are interesting, as treatment with these compounds has been shown to alter the position of some cleavage planes, presumably through their effect on the egg membranes, and on the mechanism(s) by which the spindles are positioned (Tanaka, 1976; Dan, 1979; Filosa *et al.* 1985; Langelan & Whiteley, 1985; Schroeder, 1987).

The maternal polarization in the A–V axis clearly involves a spatial localization of factors that affect cell fate, as shown by the simple test of isolating cells, or layers of cells from each region, and observing distinctions in their capacities for differentiation in culture. Only micromeres derived from the vegetal pole or their descendants can express skeletogenic genes in culture and, as discussed below, it is so far only for these vegetal-most blastomeres that a convincing argument can be made that differentiation is autonomous and depends exclusively on their inheritance of maternal factors. However, many other regional differences in cell fate have been observed along the A–V axis. Cultured vegetal half embryos may sometimes form complete larvae, while isolated animal cell caps only form ciliated epithelial balls under the same conditions (Hörstadius, 1973, pp. 45–50; Livingston & Wilt, 1989). Recognizing that the 3rd and, in the vegetal half, the 4th and 6th cleavage planes are perfectly orthogonal to the A–V axis, Hörstadius, following earlier workers, regarded the late cleavage embryo as a series of

horizontal blastomere tiers. From the vegetal pole upward these are the micromeres, veg_2, veg_1, an_2, and an_1, though the latter two are difficult to distinguish in life because of the irregular orientation of the late cleavages in this region in at least some species, and in the following are often considered together as the 'animal cap.' The position of these tiers, their composition in respect to the cell lineage, and the territories to which they give rise, are also indicated in Fig. 1. When isolated and cultured, veg_2 produces a ciliated embryoid with a large archenteron and minute skeletal elements; veg_1 a ciliated blastula which may or may not contain a rudimentary archenteron, and no skeleton; while the animal cap produces neither skeleton nor gut (Hörstadius, 1935). These deficient monsters all serve as superb indicators of inductive competence and plasticity. In perhaps the clearest and most spectacular of the numerous series of transplantations carried out over the years by Hörstadius and his colleagues, it was shown that fusion of 1–4 micromeres with each of these tiers dramatically alters the morphogenetic outcome (reviewed by Hörstadius, 1973, pp. 47–60). Thus the isolated animal cap can with the addition of micromeres produce a complete larva including gut and skeleton (the latter derived from the transplanted micromeres); veg_1, with implanted micromeres, gives rise to a larva that while deformed, nonetheless contains a prominent gut; and implantation of micromeres into veg_2 results in an exogastrula consisting of archenteron, ectoderm and mesenchyme, but no skeleton. If implanted in ectopic positions on a whole embryo, e.g. between veg_1 and an_1, micromeres *induce* the invagination and differentiation of a secondary gut. These and other similar experiments show (a) that micromeres have the capacity to induce gut formation in any tier of blastomeres with which they are placed in contact, and that probably all blastomeres in the cleavage stage embryo (except other micromeres) are competent to respond to micromere induction; and (b) that the fate of all the lineage founder cells (except the micromeres) remains plastic (as also shown by changes in cell fate induced by LiCl treatment, discussed later). Similarly, (c) implanted veg_2 cells also induce *adjacent* an_2 cells to participate in gut formation (Hörstadius, 1973, p. 68–69). Therefore, (d) amongst the inductive actions of micromeres is *repression* of this capacity of veg_2 cells to induce veg_1 cells to form endodermal structures. Another capacity of veg_2 progeny that is normally suppressed by the presence of micromeres and their derivatives is the formation of skeleton from the secondary mesenchyme that is delaminated from the archenteron during gastrulation (Driesch, 1893; Ettensohn & McClay, 1987). In chimeric and partial embryo combinations, whatever cells give rise to gut and thence secondary mesenchyme produce skeleton as well if the primary skeletogenic mesenchyme of micromere origin is missing or deleted. Since this repression may be demonstrated as late as postgastrular stages (Ettensohn & McClay, 1987), it probably is not to be included in this list of *cleavage stage* inductive effects. Since, when veg_2 cells are implanted on the vegetal surface of the animal cap, embryos form that contain complete aboral ectoderms, it may also be concluded (e) that veg_2 cells can induce adjacent blastomeres to perform the functions of veg_1, which normally gives rise to large portions of the aboral ectoderm (Fig. 1). Finally, implantation of the veg_1 tier on the vegetal surface of the animal cap causes the differentiation of oral structures (mouth and ciliated bands) which usually do not form from isolated animal caps (occasional exceptions may be due to an unusually low 3rd cleavage plane; Hörstadius, 1973, pp. 47–48, p. 43). Since, as shown in Fig. 1, veg_1 contributes hardly at all to oral structures (except for a small patch of supraanal ectoderm), this shows (f) that oral differentiation from the descendants of the overlying No and the two NL blastomeres is inductively dependent on veg_1 cells. By implanting micromeres at successively later times into isolated animal caps, Hörstadius also demonstrated (g) that inductive competence of animal cap cells to respond by expressing the functions of veg_2 cells, etc. persists only during cleavage (some species differences in this parameter are reported; Hörstadius, 1973, pp. 84–91). In sum, the conclusions (a–g) constitute an argument that the micromeres and the veg_2 and veg_1 tiers all possess specific inductive capacities; and that the veg_2 tier, veg_1 tier and animal cap cells are all competent to respond to the inductive signals during cleavage. Though these inductive capacities have been revealed through construction of ectopic interblastomere relationships, they must be utilized in the specification of founder cell fate in the normal undisturbed embryo.

Although Hörstadius (1939, 1973) also relied extensively on induction as an explanation for many specific experimental results, his major interpretative assumption was the double gradient theory of Runnström (1929). This idea provided an explanation for regulative reorganization along the A–V axis in experiments carried out with partial or chimeric embryos. Regulative behaviour in the sea urchin embryo is taken up later in this essay, but it is appropriate here to list briefly some of the difficulties entailed in the proposition that cell fate is controlled in the cleavage stage sea urchin embryo by diffusion-driven morphogen gradients emanating from animal and vegetal poles. First, there is little clear evidence for a positive animal pole 'influence' equivalent to that demonstrably exercised by vegetal pole cells (i.e. micromeres and veg_2 cells). The arguments classically adduced are largely 'quantitative,' i.e. when too many animal cap cells are combined with too few vegetal cells respecification in embryo chimeras is incomplete. In terms of inductive interactions a trivial explanation seems quite likely: Most of the animal cap cells are in these cases not in contact with the inducing cells. Furthermore, (a) the double gradient theory requires polar sources of inducing principles, and yet removal of the polar an_1 cells and/or micromeres does not prevent development. (b) While the animal cap cells are evidently completely pluripotential and plastic the veg_2 cells (and micromeres) are not, since they cannot be converted to ectodermal cell fate by removal of increasing amounts of ectoderm-producing cells; that



is, no endoderm cells can be induced by these operations to contribute to the ectoderm, though normal presumptive ectoderm is easily induced to contribute to endodermal structures. This was shown by vital staining experiments (Hörstadius, 1973, p. 67). (c) Supernumerary animal caps fused on top of an embryo do not affect the fate of vegetal cells in chimeric recombinations, contrary to expectation from the gradient theory (Hörstadius, 1973, pp. 69–71). In addition, (d) there are the many sharp divergences in cell fate between specific contiguous sister cells at given cleavages that are revealed in the lineage map for normal development. Particular examples include the divergence of the skeletogenic mesenchyme from the small micromere lineages (of which classical authors were unaware) at 5th cleavage; the divergence of ectoderm *vs.* vegetal plate lineages at 6th cleavage (i.e. the veg_1–veg_2 separation); and the oral *vs.* aboral separations in the VL and NL lineages (see Fig. 1). These demarcations, which account for the lateral 'bulge' in aboral ectoderm territories shown in Fig. 1, and which could not have affected the conceptions of classical authors since these lineage relations were only recently clarified, do not immediately lend themselves to an interpretation based on the

Fig. 5. Schematic diagram of proposed specification mechanism. (A) Normal process of specification in the A–V axis. At left is the zygote prior to cleavage. Dashed lines indicate the future horizontal cleavage demarcations between the layers identified in the centre diagram, which represents an embryo after 6th cleavage. The blastomere tiers of the centre diagram are labelled after Hörstadius (cf. Fig. 1, upper right). The small circles represent regulatory factors. Open and closed yellow circles are respectively inactive and active, positive regulatory oral ectoderm factors; open and closed red circles are inactive and active positive factors for skeletogenic mesenchyme; open and closed blue circles are inactive and active factors for vegetal plate (gut and secondary mesenchyme) differentiations. Two aboral ectoderm factors are portrayed that function synergistically (see text): constitutively active positive factors figured as closed green circles, and a negative factor which if in an active state, closed black circles, precludes aboral ectoderm gene expression, and if inactivated, open black circles, that permit this set of gene expressions (see text). Active skeletogenic factors are primordially localized in the vegetal region of the unfertilized and fertilized egg, and active aboral ectoderm factors are radially distributed throughout the cytoplasm that will be occupied by all ectodermal (oral and aboral) territories, i.e. an_1, an_2, and veg_1. All other factors shown are initially inactive, and globally distributed above the future micromere level in the zygote. The assignment of all other factors shown as positive in function is arbitrary, except for the aboral ectoderm (for this see text). Not shown but probably present are activators required for primary ectodermal differentiation, that are initially distributed throughout the animal cap region, veg_1, and perhaps even into the veg_2 layer (since isolated veg_2 layers produce some 'ectoderm' (see text)). These factors would account for the general ectoderm properties that are displayed by progeny of these cell layers, and that are included in their 'default' potentialities when they are cultured in isolation (e.g. formation of ciliated epithelium; see text). Vertical arrows indicate tier-specific, membrane-tethered inducing ligands; red, micromere inducers required for veg_2 function; blue, veg_2 ligands required for veg_1 functions; yellow, veg_1 ligands required for oral ectoderm differentiation. Although the classical evidence supports only a veg_1 inductive function on the presumptive oral ectoderm, the yellow arrow emanating from an_2 is inferred ad hoc because an_1 cells do not form oral structures autonomously. Changes from inactive to active states of regulatory factors are envisioned as the consequence of signal transductions mediated by the specific receptors (which are globally distributed; see text), when bound by the tier-specific ligands. At the right are the territories of the early blastula, excluding the small micromeres. Yellow shading represents the oral ectoderm territory after specification and commitment; green, the aboral ectoderm; blue, the vegetal plate endomesoderm; red, the skeletogenic mesenchyme (small micromeres are omitted for simplicity, and because they perform no known function during embryogenesis). (B) Regulative development along the A–V axis. Symbolism as in A; at left fusion of micromeres with animal cap; in centre and right animal cap treated with LiCl. The initial effect of LiCl is envisioned as short circuiting the inductive system, thus activating factors that transform the vegetal-most cells into cells that have the *inducing functions* of micromeres (or veg_2 cells). That is, the LiCl is imagined to act on the cells whose surfaces were originally internal. In both examples of regulation (left, centre) the result is recreation of the normal interacting array shown in A, centre. (C) Normal specification of O–Ab axis. An initial cytoplasmic polarization shown at left as striping (see text), centred on the future oral quadrant, has the effect of causing inductive ligands to be mounted that in the adjacent aboral quadrant results in *in*activation of the negative aboral ectoderm regulator (closed to open black circles). Result, seen from top, is to produce oral ectoderm specification in upper (i.e. those closer to animal pole) tier of cells (right) descendant from No, and the right and left NL cells, and aboral specification in the Na cell domain. Seen from side, at later stage, ~6th cleavage, secondary inductions are shown. These are, yellow arrows, induction of oral ectoderm by veg_1 cells, as in A, and black arrows, continued inactivation of negative aboral ectoderm regulator in lower NL descendants and in VL (see text). Symbolism: ap, animal pole; cb, the future ciliary band region of oral ectoderm, which appears to arise inductively at interfaces between oral and aboral ectoderm, and is ultimately a site of neurogenesis. Lineage designations in parentheses indicate *descendants* of the indicated cells at the stage shown (see Fig. 1 for exact designations at 6th cleavage).

shapes of axially oriented gradients.

Since the cell boundaries demarcate the domains of cell fate in all of these cases, the cell fate geometry is much more easily accommodated by a process in which the cell boundaries *per se* participate, e.g. as sites where are located receptors for inducing ligands. (e) Direct studies of the effect of cell interactions on expression of various molecular markers in cultures of dissociated embryo cells show that contact between cleavage stage blastomeres decisively influences gene expression (except in the skeletogenic mesenchyme), including lineage-specific gene expression (see above; D. L. Hurley, L. M. Anger & R. C. Angerer, pers. commun.; L. Stephens, T. Kitajima & F. Witt, pers. commun.). Finally, perhaps a prejudice rather than an argument, Hörstadius' (and others') application of gradient theory to his own experiments leads to 'explanatory' concepts that are not easily put to scientific test, such as 'the battle between animal and vegetal forces... the struggle and defeat of vegetal power,' 'regulation towards harmony,' etc. On the other hand, cell-to-cell inductive system suffices as an explanatory framework for the experimental results (Davidson, 1986, pp. 501–503; Wilt, 1987) and, as proposed below, can potentially be applied to cases of regulative respecification in this embryo.

The development of the oral–aboral (O–Ab) axis provides additional insight. There is no reliable evidence whatsoever that the second axis is preorganized in the unfertilized egg as is the A–V axis. Were there even a 'tendency' toward such a preorganization in the unfertilized egg it must be so labile that it can easily be reversed by exactly the same sorts of experimental operations that demonstrate the complete stability of the primordial A–V axis. For example, meridional partial or fused embryos from early to late cleavage

stages all can respecify the O–Ab axis and form complete pluteus larvae (many examples are reviewed by Hörstadius, 1973, pp. 96–102), while transverse partial embryos never accomplish respecification of the A–V axis. Nor can the latter be disturbed even by centrifugation that redistributes visible cytoplasmic organelles (Morgan & Spooner, 1909; Hörstadius, 1973, pp. 81–84). Yet Czihak (1963) showed that by 3rd cleavage (in undisturbed embryos) the future oral pole of the O–Ab axis is already marked by a cytoplasmic polarization that can be identified by redox stains, and the fluoresceinated dextran lineage studies of Cameron *et al.* (1987) demonstrate that the polar animal half blastomeres of the 8-cell embryo in this axis are clonal founder cells, respectively giving rise exclusively to oral and aboral ectoderm. Recent experiments of R. A. Cameron *et al.* (unpublished) in which one blastomere of 2-cell embryos is labelled with a fluorescent lineage tracer show that in at least 90 % of cases the O–Ab axis is formed about 45° counterclockwise from the plane of 1st cleavage (as viewed from the animal pole). Many classical studies show that the sperm may enter anywhere with respect to the O–Ab axis (reviewed by Hörstadius, 1973, p. 96; Schatten, 1982), and hence this relation between the plane of first cleavage and the O–Ab axis is the earliest indication of a possible causal process. That is, the cleavage plane *per se* or the cytoskeletal asymmetry that positions it, might also locate and orient the cytoplasmic polarization that begins the process of O–Ab axis specification. In any case, by the second cleavage, which is orthogonal to the first and is also vertical, the polar oral and aboral domains of the second axis are established. Fig. 1 shows how progressive separation of oral and aboral domains occurs in the progeny of the lateral NL and VL cells during the next several cleavages. Since all of this can be repeated anew in, for example, the four blastomeres of a dissociated 2nd cleavage embryo, or in fused meridional half embryos, it is clear that, first, the initial polarization is labile and can be reestablished and, second, the polar cells of the newly defined O–Ab axis must then be able to respecify their own lateral neighbours. Thus, whereas the initial polarization in the A–V axis is accomplished during oogenesis, that of the O–Ab axis occurs after fertilization, but in both axes the functional identity of many of the founder cells formed during cleavage seems obviously to depend on intercellular inductive interactions.

In summary, the way in which the invariant cell lineage of the early sea urchin embryo contributes to the progressive spatial organization of cell fate can be envisioned as follows. The initial A–V polarization of the egg must involve localization of some factors that affect cell fate in blastomeres inheriting them, since the 'default' developmental capacities of cells or cell tiers isolated from various positions along this axis differ. Thus factors required for skeletogenesis are localized at the vegetal pole, and, if vegetal tiers of cells are cultured, the progeny of the blastomeres included can interact to form gut, which the progeny of animal tiers of cells cannot, etc. To this extent there is an initial 'gradient' of morphogenetic capacity. Cleavage is the mechanism by which the egg is divided into three-dimensional domains of cell fate, most of which greatly exceed in diversity the 'default' potentials that can be accounted for by the primordial pattern of organization along the A–V axis. All O-Ab differentiations are specified after fertilization. The order of the cleavage planes is probably not important, as it can be perturbed, e.g. by application of pressure (Driesch, 1892) nor is their exact position, as shown by successful development in centrifuged and detergent-treated eggs. However, *in the undisturbed egg*, the cleavage pattern is rather rigidly specified, probably a simple consequence of the means by which it is encoded in the cytoarchitecture of the egg. It is argued here that the specification of *founder cells in the sea urchin embryo is largely due to* intercellular inductive interactions, along *both* A–V and O–Ab axes. Since *founder cells* are in the cases of the earliest differentiations, e.g. aboral ectoderm, equivalent to future *gene regulatory domains*, these inductive interactions must determine the specific sets of genes that are differentially expressed in the clonal progeny of the founder cells. Thus the role of cleavage in the normal undisturbed embryo could be to position the boundaries of the regulatory domains, and to provide the cell membranes where the specifying inductive interactions take place.

II. Embryonic expression of CyIIIa and SM50 fusion genes injected into the egg, and molecular interactions of their *cis*-regulatory regions

We turn now to some recent experiments in which regulatory elements of the aboral ectoderm marker gene CyIIIa and the skeletogenic mesenchyme marker gene SM50 have been introduced into unfertilized eggs, and found to direct appropriate spatial and temporal expression in the early embryo. By this route we proceed from the spatial process of territory foundation to the molecular mechanisms by which regional patterns of gene expression are initially instituted. In general terms the following results require that the spatial activation of the CyIIIa and SM50 genes, i.e. their lineage specificity, is achieved by interaction of the regulatory DNA with diffusible factors, and so also is the timing of their expression. Therefore, since the exogenous DNA is incorporated in the blastomere nuclei in ectopic positions, neither inclusion of the gene in specific 'chromatin domains' nor any form of 'imprinting' during gametogenesis, nor regulatory sites located at very great distances from the transcriptional initiation points could be necessary for correct expression. The conclusion supported by these experiments is thus that the different cytoplasmic regulatory domains set up during cleavage in the sea urchin embryo owe their specific functional characters to their diverse contents of active gene regulatory factors. As summarized in the following, there is now extensive evidence that there are diffusible DNA-binding proteins that interact specifically at multiple sites in the upstream regions of the CyIIIa and SM50 genes.

1. *The CyIIIa gene*

The fate of the cells whose gene copy will express CyIIIa is plastic, as reviewed above, and the evidence suggests an inductive component in the mechanism responsible for CyIIIa activation. Gene transfer experiments that assess CyIIIa regulatory sequence function *in vivo* have revealed an enormously complex and interesting *cis*-regulatory domain that is required for activation. This extends about 2·5 kb upstream from the transcriptional initiation site of the CyIIIa gene, and includes no less than 15 sites where high specificity interactions with nuclear proteins have been demonstrated. A diagram describing the fusion gene construct in which the coding region of the CyIIIa actin gene, save for the initial ten amino acids, is replaced by that of the CAT (bacterial chloramphenicol acetyl transferase) gene, is shown in Fig. 2A; and in Fig. 2B can be seen the location of the known sites of DNA-protein interaction.

i. *Gene transfer: CyIIIa regulatory functions* in vivo

For tests of regulatory function *in vivo* the linearized fusion constructs are injected into unfertilized (for *S. purpuratus*) or fertilized (for *L. variegatus*) egg cytoplasm by methods developed and characterized several years ago in our laboratory (Flytzanis *et al.* 1985; McMahon *et al.* 1985; Franks *et al.* 1988*a*). Within minutes after its introduction the exogenous DNA is rapidly ligated into one or a very small number of large concatenates, and these are taken up into the blastomere nuclei during early cleavage. The DNA is *stably* incorporated, replicates at the rate of the genomic DNA in the nuclei in which it resides throughout embryonic and larval development, and we do not observe the subsequent loss of most exogenous sequence that is widely reported to occur in late *Xenopus* embryos after cytoplasmic injection of DNA (Rusconi & Schaffner, 1981; Etkin *et al.* 1984; Etkin & Pearman, 1987). In 75 % of embryos that retain any detectable exogenous DNA (i.e. ~80 % of all eggs into which it is injected), the exogenous DNA appears to be stably incorporated, i.e. probably integrated in the genomic DNA of a single 2nd, 3rd, or 4th cleavage blastomere nucleus (Hough-Evans *et al.* 1988) and, in the remainder, at later cleavages. However, integration has been directly proved, by cloning out the junctional sequences from genomic DNA preparations, only for postmetamorphosis juveniles raised from injected eggs (Flytzanis *et al.* 1985). The amount of amplification of the exogenous DNA thereafter, to a first approximation, thus depends simply on how soon stable incorporation occurs (i.e. for a 2nd cleavage incorporation it would participate in ~7/9 cleavage replications, but for a 4th cleavage incorporation only 5/9 cleavage replications; see Franks *et al.* 1988*a*; Hough-Evans *et al.* 1988). The consequence in any case is that stable incorporation is mosaic, and the fraction of embryo cells that is likely to retain the exogenous DNA ranges from a few percent to ~25 %. However, as shown directly by RNA–DNA *in situ* hybridization (Hough-Evans *et al.* 1987) there is no bias in respect to the lineage element in which incorporation occurs, or to the cell types in advanced embryos in which it is retained.

The CyIIIa·CAT fusion shown in Fig. 2A is expressed appropriately, both in temporal and spatial terms, when introduced into unfertilized eggs by this method. Thus CAT enzyme activity appears abruptly in late cleavage (Flytzanis *et al.* 1987) when the CyIIIa gene is normally activated (see above). Similarly, CyI·CAT (Katula *et al.* 1987) and Spec 1·CAT (W. Klein, pers. commun.) fusions have also been shown to be activated on the correct developmental schedule after injection into unfertilized sea urchin eggs. *In situ* hybridization using CAT antisense RNA probes demonstrates that in embryos bearing CyIIIa·CAT, CAT mRNA appears only in ectoderm cells, and where it is possible to distinguish oral from aboral ectoderm in given sections, only in aboral ectoderm cells (Hough-Evans *et al.* 1987, 1988; Franks *et al.* 1988*b*; unpublished data). When present in any other cell types the CyIIIa·CAT gene remains silent. We have at this writing observed complete serial sections or partial sections of >300 embryos raised from eggs injected with CyIIIa·CAT from many independent experiments, and have never seen CAT mRNA expressed in gut or mesenchyme cells.

In most of the experiments reviewed here the measured number of CyIIIa·CAT genes actually incorporated in the embryo nuclei is in excess of the number that can be serviced by the specific transcription factors required. This is shown by the fact that for greater than several thousand genes per nucleus the amount of CAT activity per gene declines in inverse proportion to the number of exogenous genes (Flytzanis *et al.* 1987); and by direct demonstration of functional saturation, as the number of exogenous CyIIIa·CAT genes incorporated is increased. A representative saturation experiment (Livant *et al.* 1988) is reproduced in Fig. 3A. At saturation the number of CyIIIa·CAT mRNA molecules in cells of 24 h embryos bearing the exogenous fusions, as estimated by probe protection (Flytzanis *et al.* 1987; Hough-Evans *et al.* 1987), is probably ~20× the number of endogenous CyIIIa mRNAs per aboral ectoderm cell [Lee *et al.* (1986) measured ~200 CyIIIa mRNAs per expressing cell]. Calculations based on the relative stability of CAT mRNA and CyIIIa mRNA (Flytzanis *et al.* 1987; Lee, 1986) suggest that in the initial linear portion of the saturation curve the productive transcription rate of the CyIIIa·CAT genes could be within a factor of two of that of the CyIIIa genes in normal embryos. Thus it is unlikely that any significant positive regulatory elements are missing from the CyIIIa·CAT fusion construct. The main point is that saturation of expression implies that regulatory sites in the many incorporated exogenous genes are competing with one another for diffusible factors present in limiting concentration. This is demonstrated directly in the experiment shown in Fig. 3B (Livant *et al.* 1988). Here the regulatory domain (*Sph*I–*Sal*I site; see Fig. 2A) was coinjected in various low molar excesses as competitor with the whole CyIIIa·CAT construct, and it can be seen that CAT activity falls off

at a rate that is within a factor of about two of stoichiometric prediction based on the molar ratios of competitor to CyIIIa·CAT molecules. We can now extend this approach to test for individual sites where necessary positive regulatory interactions occur, by coinjecting molar excesses of small fragments (ligated into long carrier DNA molecules) that contain one or another of the specific binding sites shown in Fig. 2B (Franks *et al.* 1989; unpublished data). Examples are shown in Fig. 3C–E. In Fig. 3C and D it can be seen that low molar excesses of fragments including the P4 and P5 binding sites, respectively (see Fig. 2B), each severely depress CyIIIa·CAT activity, demonstrating that both of these regions contain essential positive regulatory sites bound by factors of limited availability. In contrast, molar excess of the P3a site does not result in significant competition, as shown in Fig. 3E. In all, seven independent DNA-binding regions have been shown to compete, more or less equivalently, i.e. within a factor of about three of the competition obtained with the complete regulatory domains. Thus, simultaneous interactions at all of these regions must be required for maximal function, though we do not yet know in most cases whether exactly the same sequences within the coinjected fragments as are involved in DNA–protein interactions *in vitro* are required for the observed competitions *in vivo*. The regions displaying effective competition are those bound by the putative factors (or sets of factors) designated P1, P2, P3B, P4, P5, P7I, and P8 (Franks *et al.* 1989; unpublished data). The DNA sites at which interaction with proteins occurs (*in vitro*) in these regions of the regulatory domain are all distinct from one another, and these proteins appear to be present in different amounts, and to display different DNA binding characteristics (Calzone *et al.* 1988; see below); thus pending their purification, it is necessary to assume that each of the competitor regions reacts with a *different* regulatory factor. No significant competition was observed with fragments bearing the sites for P3A, P7II, or P6. While this could in principle be due to high prevalence of the respective factors, the *in vitro* measurements reviewed in the following show that this is an unlikely explanation, since, for example, the P2 protein is among the most prevalent, and yet *in vivo* this binding region displays clear competition with coinjected CyIIIa·CAT.

Samples of embryos used for these competition experiments were also sectioned and surveyed by *in situ* hybridization for the spatial localization of CAT expression (B. Hough-Evans *et al.* unpublished data). The object of this exercise was to screen for a possible *negative* spatial control element, on the principle that competitive binding of a factor of such function might cause ectopic expression. A negative component of the control system responsible for lineage specification of CyIIIa expression was suspected because of the result reported earlier by Franks *et al.* (1988*b*) in which CyIIIa·CAT expression was shown to occur in gut, mesenchyme and oral ectoderm, as well as aboral ectoderm, when introduced into eggs of a distantly related sea urchin species, *Lytechinus variegatus* (recall that the CyIIIa gene was isolated from *S. purpuratus*). The simplest interpretation of this result, though clearly not the only one, is that a DNA–protein interaction normally responsible for negative spatial control (i.e. keeping the gene off in gut and mesenchyme, etc.) had failed because of evolutionary divergence between the necessary CyIIIa regulatory site(s) and the respective binding factor(s). Preliminary data show that two of the CyIIIa regulatory fragments so far studied by *in vivo* competition do in fact cause ectopic expression. When excess fragments containing the P3 or P7II binding sites are coinjected with CyIIIa·CAT, expression is observed in gut and/or mesenchyme in most embryos, something never seen under normal conditions, or in competition experiments with any other of the fragments tested.

ii. In vitro *analyses of DNA–protein interactions in the CyIIIa regulatory region*

The 15 specific sites indicated in Fig. 2B have been mapped to the nucleotide level with conventional methods (Calzone *et al.* 1988; N. Thézé *et al.* unpublished data). Quantitative analyses of the gel shift data show that the affinity of the DNA-binding proteins in the embryo extracts for the CyIIIa site mapped in Fig. 2B range from $\sim 10^4$ to over $10^6\times$ their affinity for synthetic or random DNA sequences (Calzone *et al.* 1988). In 24 h embryos, a stage at which the CyIIIa gene is active, the factor concentrations most commonly observed are a few thousand molecules per nucleus (Calzone *et al.* 1988). These values are consonant with estimates obtained for the concentrations of limiting factors in living embryo nuclei, by means of the *in vivo* saturation experiments, such as that shown in Fig. 3A (Livant *et al.* 1988). With one exception, all of the high specificity DNA-binding factors that can be detected *in vitro* (i.e. those mapped in Fig. 2B) appear only after fertilization. Significant quantities of a factor binding to the P5 site can alone be identified in unfertilized egg extracts (though the gel shift migration of the complex formed by this maternal factor differs from that formed by the factor binding to the same sequence that is present in embryo nuclear extracts). By 7 h postfertilization (midcleavage), all of the factors identified can be detected in nuclear extracts. These could be the products of translation either of maternal messages, or of zygotic transcripts. However, an interesting distinction classifies these factors into a set that displays a sharp rise in prevalence (i.e. 10- to 50-fold) between 7 h, when the CyIIIa gene is yet inactive, and 24 h, when it is being expressed in aboral ectoderm nuclei; and a set that is present in about the same concentration at 7 h and at 24 h (Calzone *et al.* 1988). Calculations suggest that for those factors present at low levels at 7 h the CyIIIa binding sites would not be appreciably occupied *in vivo* until levels approaching or exceeding those measured in the 24 h nuclei are attained. The rise in concentration of the proteins (or of modified, DNA-binding forms of preexisting proteins) could constitute causal elements of the mechanism controlling *temporal activation* of the CyIIIa gene, particularly in cases where the *in vivo*

competitions have indicated that the interaction is required for gene expression. Factors that are present at their final levels early and that are also required for expression, could serve as general enhancer proteins, but another more interesting possibility must also be kept in mind. Since aboral ectoderm founder cell specification occurs during cleavage, prior to activation of the CyIIIa gene, any regulatory proteins that are directly affected by this event would have to be present during cleavage. Such factors could include the molecular control elements directly responsible for lineage-specific, spatial patterns of expression.

As an initial scan for CyIIIa regulatory sites that may function in spatial control we have carried out intergenic gel shift competitions *in vitro*, using as competitor regulatory regions from three other sea urchin genes that display either the same or different patterns of expression (P. Thiebaud *et al.* unpublished data). Such experiments detect functionally homologous DNA-binding sequences *in vitro*, under gel shift conditions, in which, as above, only highly specific interactions are observed. The genes from which the competitor regulatory sequences tested were derived are: the Spec 1 gene, which as noted above is also expressed only in aboral ectoderm; the CyI gene, which is expressed everywhere in early embryos, and later only in gut and oral ectoderm (Cox *et al.* 1986); and the SM50 gene. Regulatory regions of these genes have been identified by gene transfer experiments (for Spec 1, W. Klein, pers. commun.; for CyI, Katula *et al.* 1987; for SM50 see below). An aboral ectoderm, battery-specific spatial control element might be expected to be shared by Spec 1 sequences, while a temporal regulation site, or a general enhancer might occur in any of this set of genes, since they are all activated at about the same time, late in cleavage. Perhaps the most interesting of the results obtained in this series of experiments has been that certain upstream fragments of the Spec 1 gene do indeed compete very strongly for given nuclear proteins with three of the putative CyIIIa regulatory regions, those containing sites for P1, P2, and P7II (that is, the Spec 1 fragments have 0·5–10× the affinity for these respective proteins as do the cognate CyIIIa sites). No sequences in the necessary and sufficient regulatory domains of the SM50 or CyI genes compete with these particular CyIIIa sites. Spec 1 also contains a site that reacts with the P3A protein, a putative negative space regulator, as does the SM50 gene.

In Fig. 4 results of these various measurements are collated, and the CyIIIa interaction sites are tentatively classified according to their possible functions. While for some interactions (coloured regions) the experimental evidence suffices to justify the classifications shown at least at the level of a working hypothesis, others remain enigmatic; in general Fig. 4 is intended only to convey what seems a consistent summary as of this stage in our incomplete knowledge. Three classes of interaction are indicated in Fig. 4, *viz.* those possibly functioning as general enhancers, those possibly mediating temporal expression, and those possibly mediating spatial expression (see legend).

In summary, developmental expression of the CyIIIa gene in late cleavage is certainly controlled through multiple interactions with embryo nuclear proteins, occurring at specific locations in the extended upstream regulatory domain. A perhaps unexpected aspect is that there appear to be multiple, *different regulatory interactions carrying out each of the classes of function we can distinguish*. These are not redundant or dispensible, since the *in vivo* competition experiments show that if any of seven different interactions is prevented *in vivo* the activity of the gene is severely depressed, while interference with two other interactions causes expression in cell lineages where the gene is normally forever silent. Furthermore, the data in Fig. 4 suggests that spatial regulation could be mediated by *both* positive and negative interactions.

2. *The SM50 gene*

The regulatory biology of the SM50 gene clearly differs from that of the CyIIIa gene. Thus, activation of this gene in the skeletogenic mesenchyme lineage does not depend on intercellular interaction; the fate of cells expressing the SM50 gene is not plastic; and the cells that in the undisturbed embryo express this gene appear to be specified by their inheritance of a specific region of egg cytoplasm localized at the original vegetal pole of the egg. The most impressive modern evidence for the autonomy of skeletogenic lineage differentiation derives from the many studies showing that skeletogenesis and the associated molecular expressions occur in cultures of isolated 4th cleavage micromeres (reviewed in Davidson, 1986, pp. 220–228), and from the studies of Stephens *et al.* (pers. commun.) cited earlier, which show in particular that SM50 gene expression achieves at least 50% of the normal level in descendants of blastomeres separated from one another as early as the 4-cell stage, and maintained under culture conditions that preclude any intercellular contact thereafter (see also similar results of Hurley *et al.* pers. commun.).

Exogenous fusion constructs in which the CAT reporter is placed under control of the SM50 regulatory domain (see Fig. 2C) and injected into unfertilized eggs function properly, just as do the CyIIIa fusions (Sucov *et al.* 1988). Thus, in embryos derived from eggs cytoplasmically injected with SM50·CAT, CAT transcripts are almost exclusively confined to skeletogenic mesenchyme cells (i.e. except for a very occasional embryo displaying one or two anomalously labelled ectoderm cells). This result demonstrates that expression of the SM50 gene depends on factors that can interact with the regulatory sequences included in the fusion construct. Therefore, at least the functional forms of one or more of these factors must be specifically localized, in the undisturbed egg, to the territory occupied by the skeletogenic founder cells and their progeny. Only about 450 nt of upstream sequence (and no intron sequences) are required for high level skeletogenic mesenchyme-specific expression. A map of the SM50 DNA-binding sites within the 450 nt domains that were observed *in vitro* with embryo nuclear proteins is shown in Fig. 2D (Sucov *et al.* 1988; Sucov, 1988).

Though analysis of DNA–protein interactions in the 450 nt SM50 regulatory domain is as yet incomplete, there are at least five nonoverlapping sequences of interest. Three of these appear to function positively. These are the CCAAT site and the two 'A' binding sites. A deletion that removes 'A' sites causes expression of the CAT reporter gene to fall to a few per cent of the control value. An interesting result is that the P3A factor, which as discussed above is probably a negative regulator of the CyIIIa gene, appears to be identical to the factor that binds the 'B' site of the SM50 gene. This element does not function positively in the SM50 gene either. Note that the intergenic competition shown in Fig. 4 suggests that both genes may utilize the same factor, though the regulatory mechanisms controlling these genes are obviously different. The mRNA for this factor is present in the unfertilized egg, and displays a sharp transient increase in concentration after cleavage.

These details aside, it is clear that, just as implied by the results of the gene transfer studies, interactions of regulatory significance indeed occur in the *cis* control domain of the SM50 gene. This domain is simpler, in that it includes many fewer sites of interaction, and is much less extended than the CyIIIa regulatory domain. One wonders whether this could be an external reflection of the simpler spatial regulation biology of the SM50 gene in that this gene belongs to an autonomous rather than a conditional pathway of lineage segregation and differentiation.

III. How the sea urchin embryo may 'work': Lineage specification, and spatial organization of differential gene expression

1. *Insufficiency of a 'mosaic' interpretation*

The CyIIIa and SM50 genes are of course not the only sea urchin genes demonstrated to be controlled by interactions with regulatory factors in the early embryo. Several other genes expressed in particular spatial and/or lineage domains that have been studied by gene transfer experiments are mentioned above. In addition, convincing evidence has been obtained demonstrating that temporal control of early and late histone genes (which are expressed in dividing cells) depends on interaction of specific regulatory factors with *cis* control regions of these genes (Barberis *et al.* 1987; Busslinger & Barberis, 1985; Colin *et al.* 1988; Lai *et al.* 1988; Vitelli *et al.* 1988). Both spatial and temporal regulation would thus seem to rest causally on the presentation of appropriate regulatory factors at the right times and places. Given the invariant cell lineage and the subsequent invariant patterning of gene expression, it might be supposed that the initial set of differential gene expressions in the embryo depends simply on the sequestration of appropriate sets of maternal regulatory factors to each equivalent group of founder cells, as these are always contiguous. However, this idea, essentially a molecular version of the old theory of mosaic development, neither predicts nor accounts for the *conditionality of cell fate* in the sea urchin embryo. As we have seen, even the most rigid and autonomous of the cell fates considered, *viz.* that of the skeletogenic mesenchyme lineages, can be elicited in other regions of the embryo. Ectopic skeletogenesis by progeny of veg_2 and veg_1 cells, described by Hörstadius and his colleagues, was cited earlier. Furthermore, and perhaps even more remarkable, isolated animal cap cells which in normal development are ancestral exclusively to oral and aboral ectoderm, and to their neurogenic derivatives, if treated with LiCl give rise to pluteus larvae that include skeletal structures (see below for some details and references). Considered in terms of the reasonable inference from current results with the exogenous SM50·CAT genes that spatial sequestration of *trans-acting regulatory factors to the skeletogenic mesenchyme* might explain skeletogenic gene activation, such extreme examples of regulatory behaviour appear paradoxical. That is, the *capacity* for skeletogenesis is in fact not confined to any particular lineage element of the cleavage stage embryo, and so, seen in this light, neither should be the responsible regulatory factors.

Similar arguments apply to the O–Ab axis, with respect to aboral ectoderm-specific gene expression. Assignments in this axis remain extremely plastic and capable of axial reorientation (unlike the A–V axis) far into cleavage. Not only are all four blastomeres at 2nd cleavage able to reform complete plutei if separated and cultured (Driesch, 1891, 1900; Boveri, 1907; Hörstadius & Wolsky, 1936), but so also do meridional half embryos isolated at 4-cell, 16-cell, and later stages (Hörstadius, 1973, pp. 96–103); and if fused together meridional half eggs and meridional halves of 16-cell, or 32-cell embryos all form complete larvae with differential O–Ab structures, as do fusions of two whole 32-cell-stage embryos that had been opened and joined along the animal–vegetal axis (Hörstadius, 1973, pp. 96–104). Furthermore, treatment with Li^+ radializes sea urchin embryos if the exposure is in cleavage stages, apparently due to suppression of morphogenesis of oral structures (Hörstadius, 1973, pp. 107–111). These experiments, or an appropriate subset, clearly need to be repeated using molecular markers of oral and aboral differentiation. However, taken at face value they demonstrate overwhelmingly the plasticity of aboral specification, though by the end of cleavage this specification has been locked in, and is manifested as a distinctive set of differential gene expressions. The *capacity* to produce aboral ectoderm evidently remains radially distributed beyond 6th cleavage, even though a predictive cytoplasmic polarization can be observed much earlier, i.e. by 3rd cleavage (Czihak, 1963; see above). Thus it is again obviously an insufficient conception to imagine that the factors responsible for aboral ectoderm gene expression are simply sequestered on one side of the embryo as a consequence of this initial polarization, whatever its origin.

2. *Mechanism of inductive lineage specification: A theory that explains regulative capacity*

With particular reference to those aspects of early sea

urchin development described here, the specification of the 'invariant but conditional lineages' of this embryo might operate as follows:

i. *Conditional specification of lineage founder cells*

It is envisioned that this process is mediated by inductive intercellular interactions occurring during cleavage, i.e. when specification takes place. In the theory the causal inductive ligand–receptor interactions would occur at the founder cell membranes, and would activate signal transduction mechanisms, the result of which would be to modify DNA-binding factors (or their cofactors), changing their functional state from transcriptionally inactive to active (or *vice versa*). [For examples of specific gene regulatory factors that are altered in their state of activity by modifications such as might be (or are known to be) mediated by signal transduction systems see, for instance, Chiu *et al.* (1988) and Lamph *et al.* (1988), for the c-*fos*/AP-1 complex; Prywes & Roeder, 1986, for a factor binding to an enhancer of the c-*fos* gene that is activated by EGF; Sorger *et al.* (1987), for heat shock transcription factor; Zinn *et al.* (1988) for a factor binding to the β-interferon gene, the activity of which appears to depend on a protein kinase; Baeuerle & Baltimore (1988) for alterations in NF-κB activity dependent on lymphocyte stimulation.] *Inductive alteration of regulatory factors is envisioned as the general mechanism by which cytoplasmic domains formed by the intersections of cleavage planes, and defining given founder cell territories, are converted to the appropriate gene regulatory domains.* It would follow that such DNA-binding factors as are capable of spatial control of gene expression, are in their premodified (inactive) forms, distributed far more widely in the embryo than are the ultimate patterns of expression that they mediate. The distribution of these activatable factors would account for the distribution of regulative potentialities amongst the embryonic blastomeres.

ii. *Specification of autonomously differentiating cell lineages, and of specific functions expressed autonomously in cells derived from given regions of the egg*

Gene regulatory agents required to activate autonomous functions that are expressed irrespective of cell interaction would by the onset of cleavage be localized in the cytoplasm inherited by the appropriate founder cells, in a form that is already functional. That is, no further modifications would be required. However, inactive forms of the same factors may be, and in the sea urchin embryo (see below) in some cases must be, very widely or even globally distributed. Localized active regulatory factors, together with more widely dispersed inactive forms, are not inconsistent. As a purely hypothetical example, during oogenesis a regulatory gene might produce mRNAs and proteins that are not localized, but modifying enzymes bound to the cytoskeleton at the future vegetal pole might change to an active state those molecules trapped in this particular region of the oocyte cytoarchitecture.

iii. *Maternal and zygotic regulatory genes required for spatial expression*

Two reasonable assumptions are that the embryo will use *zygotic expression* of spatial regulatory genes to ensure the presence or appropriate concentration of regulatory products only in certain cells; and that the inductive interactions required for specification terminate during cleavage (see above). The genes coding for regulatory agents might in general respond to their own products [see e.g. Hoey & Levine (1988) for reaction of *eve* product with its own regulatory sites; or Biggin & Tjian (1988) for reaction of *Ubx* protein with a region of the Ubx transcription unit]. Thus the initial, globally or widely distributed regulatory product might be of *maternal* origin, while *zygotic* expression of the same regulator would as a result of positive feedback be confined to the appropriate lineages, wherein the product is active ('commitment'). Other kinds of maternal regulatory factors that may service genes not expressed in spatially confined domains (as well as genes that are) must also be present, e.g. general enhancer proteins, and those factors responsible for the $\geqslant$90% overlap between the set of genes expressed in the oocyte and in the early embryo (Galau *et al.* 1976; Hough-Evans *et al.* 1977; Flytzanis *et al.* 1982).

iv. *Separate temporal and spatial regulatory interactions*

At least some factors that rise in concentration late in cleavage are involved in activation of quiescent genes and do not affect spatial regulation. The CyIIIa data reviewed suggest the generalization that temporal regulatory gene products and spatial regulatory gene products will be in many cases separate, and that they interact at distinct *cis* binding sites within the regulatory domains of given genes. Therefore the *cis* regulatory apparatus must be able to assess the binding states of multiple sites, no doubt by factor interaction (Ptashne, 1986), since developmental regulation apparently depends in cases such as CyIIIa on many independent interactions.

v. *Specification along the A–V axis*

The foregoing propositions, combined with the conclusion drawn earlier that three different inductive interactions must occur along the A–V axis, lead to the following image of specification of conditional lineage elements. The vegetal polar region of the egg is considered *primordially specified*, and the relevant consequence of the state of differentiation manifested by the cells deriving from it, *viz.* the micromeres, would be their presentation of a unique set of inducer ligands here visualized as tethered to the cell membranes. Receptors for these ligands must be globally distributed in the membranes of the cleavage stage embryo, since, as pointed out above, all or most blastomeres appear competent to respond as do veg_2 cells in the undisturbed embryo, if micromeres are implanted next to them (see above). Veg_2 properties would be programmed by the activation of latent gene regulators in response to micromere induction, although veg_2 cells may have the

capacity in the absence of the repressive influence of micromeres to carry out these same functions autonomously. The interface between the veg_2 and veg_1 tiers is a sharp cell fate divide, in that in the normal undisturbed embryo veg_1 cells display exclusively ectodermal fates while no cells of veg_2 do so (see Fig. 1). Thus the inducing ligands displayed on the upper surfaces of veg_2 cells would affect regulators specifying particular ectodermal functions in the pluripotent veg_1 cells, perhaps by permitting the oral and aboral ectoderm regulators to operate (see *vi.* below). In turn inductive ligands of veg_1 cells would be required for activation by signal transduction of genes expressed in animal cap cells to produce ciliated band and stomodeum (i.e. oral structures). In general, then, each tier (micromere, veg_2, veg_1), must be polarized cytoskeletally in the A-V axis, and must display a specific set of inducer ligands upward, while the receptors for these could be globally present. In each tier except the initial micromere tier, the ligands would be synthesized, mounted in the cell membranes, or activated, or all of these, in response to the induction from the next most vegetal tier, and would determine the state of the relevant gene regulatory agents. A simple diagram is shown in Fig. 5A.

vi. *Specification along the O–Ab axis*

The initial cytoplasmic polarization that marks this axis in the undisturbed embryo is centred on the No quadrant (viewed from the animal pole) and includes portions of the cytoplasm inherited by the NL cells. An effect of this polarization could be to cause display of a membrane-bound ligand that in the adjacent cell, Na, causes a signal that inactivates a negative control factor. This would allow aboral ectoderm functions in the domain inherited by progeny of the Na cell (see Fig. 5C). Since it appears that if negative control factors are competed out or fail to function, ectopic expression of aboral ectoderm genes occurs (see above) positive control factors for these genes are pictured in Fig. 5C as constitutively active. The subsequent partition of the NL descendants (see Fig. 1) into the ancestors of ciliated band-producing cells of the oral field and into aboral ectoderm requires further interactions, including those of the cells of the veg_1 tier, one possible mode for which is suggested in Fig. 5C (see legend).

3. *Regulative development*

Classical and modern evidence indicates two major classes of experimental circumstance in which can be displayed the amazing capacities of sea urchin embryos to organize regulative corrections in development, that clearly involve changes in cell fate. These are alterations of the normal interblastomeric spatial relationships, along either axis; and treatment with LiCl. Many of the most unequivocal examples illustrating the consequence of ectopic blastomere rearrangements and isolations are mentioned above. There is a large literature (reviewed by Lallier, 1964) on the effects of LiCl and other agents which appear to cause overproduction (relative to ectoderm) of the major veg_2 product, *viz.* gut (and hence these are called'vegetalizing agents'). Only three particular experiments are here considered, of which the third provides a molecular insight into the first two: (a) The progeny of isolated animal caps (von Ubisch, 1925*a*,*b*, 1929; Hörstadius, 1936) or of pairs of sister animal cap cells (Livingston & Wilt, 1989), if cultured in a LiCl medium, develop gut and spicules, which they do not do if cultured without Li^+. Since implanted micromeres have largely the same effects on animal half embryos, e.g. induction of gut formation, Hörstadius' intuition (1973, p. 78) that micromeres and Li^+ function in a 'parallel' manner seems justified. (b) Hörstadius (1936) removed a few an_2 cells of an isolated late cleavage animal cap that had been cultured in LiCl and then implanted these cells on the (vegetal) surface of a newly prepared *un*treated late cleavage animal cap. The result was again that pluteus larvae with gut and skeleton formed, mainly from the cells of the untreated cap. This experiment shows that the *inducing* function normally exercised by the micromeres (or veg_2 cells) is caused to appear in the an_2 cells by Li^+ treatment, so that they now act as veg_2 cells or micromeres. (c) Livingston & Wilt (1989) showed that in a majority of the embryoids produced by the progeny of pairs of animal cap cells cultured in LiCl the SM50 gene is expressed, while in controls (i.e. no Li^+) this gene is not significantly expressed. So also is a gut alkaline phosphatase marker expressed after LiCl treatment. This experiment supports the inference that LiCl elicits ectopic gene expression normally found only in other lineages, i.e. that treatment with this agent indeed results in respecification at the gene level. The interpretation of the normal process of embryogenesis proposed in this paper would be applied to these experiments as follows (see Fig. 5B). Experiment B explains experiment A if Li^+ indeed substitutes for micromere (or veg_2 cell) induction. This is not a bizarre proposition since, as pointed out in related contexts elsewhere (Slack *et al.* 1988; Sucov *et al.* 1988; Livingston & Wilt, 1989), LiCl interferes with the enzymic metabolism of inositol phosphates generated in response to ligand-specific receptor interactions (e.g. see Berridge & Irvine, 1984; Thomas *et al.* 1984; Mitchell, 1986; Sherman *et al.* 1986; Hansen *et al.* 1986), and may have direct effects on certain G-proteins involved in receptor coupling as well (Drummond, 1988; Avissar *et al.* 1988). The results obtained would then be the consequence of the normal presence of inactive forms of SM50 regulators in animal cap cells (Fig. 5B) which can be activated either by inductive signal transduction (experiment B), or by the 'short circuit' of these membrane receptor compounds normally utilized, and activation of the same internal signal system by treatment with Li^+ (experiment C).

It is now possible to approach in principle the challenge of imagining how a more or less *properly formed* whole embryo could be recreated from a partial embryo, e.g. an isolated animal cap fused with micromeres on the vegetal side. The *normal* mechanism of blastomere specification envisioned here in some measure draws the fangs of this old problem. If most

lineage-specific gene regulators are initially present globally in inactive forms that can be converted to active forms by inductive signal transduction, the outline of events might be as follows (Fig. 5B). The implanted micromeres would induce the adjacent (an_2) cells to achieve veg_2 specification. This entails ultimate activation of genes required for construction of vegetal plate functions (cf. Fig. 1), and also the mounting of inductive ligands specifying veg_1 function on the upper surfaces of the newly defined 'veg_2' cells. Thus the next tier of animal cells is caused to assume veg_1 functions, and the next, the (oral) animal cap functions that require veg_1 induction (in this case the original O–Ab specifications would probably be preserved).

Further development of course requires many later inductive interactions, which must become all important for morphogenesis as the embryo advances beyond the organizational influence of the original maternal spatial coordinates ensconced in the cytoarchitecture of the egg. The most spectacular example so far uncovered in the sea urchin embryo is the compensatory conversion to skeletogenic function of secondary mesenchyme cells when given numbers of cells of the original skeletogenic lineage are experimentally removed (Ettensohn & McClay, 1988). These results imply a quantitative repression of the skeletogenic pathway in secondary mesenchyme cells by the normal skeletogenic mesenchyme.

4. *Some briefly described applications to other deuterostome embryos*

Most deuterostome forms rely predominantly on inductive interactions, and many display striking regulative abilities. Thus similar interpretations may apply.

i. *Hemichordate embryos*

The cleavage patterns of embryos of the distantly related phylum Hemichordata display an uncanny similarity to those of regularly developing sea urchin embryos in their polarity, and the animal–vegetal alternation of cleavage plane orientation, etc. (Colwin & Colwin, 1953). Colwin & Colwin (1950), and G. Freeman (pers. commun.) found that the regulatory capacities of these embryos are also similar. The point here is not this similarity *per se*, but the similarity despite the enormous difference in ultimate developmental outcome. The flexible mechanism of lineage specification associated with the archetypical sea urchin cleavage pattern may be an original deuterostome strategy. Thus Jeffereys (1986) argues that hemichordates are in fact closer to early evolutionary stem deuterostomes than are echinoderms, and that they may represent an offshoot from the most basic true deuterostome forms.

ii. *Ascidian embryos*

These embryos also display an invariant lineage, in which more elements appear capable of autonomous differentiation than in most forms. Tail muscle specification provides a particularly interesting example (Whittaker, 1973, 1983). Most of the tail muscle cells derive from cleavage stage blastomeres that differentiate autonomously, their fate apparently determined by maternal factors that are regionally segmented into the lineage founder cells. However, Deno *et al.* (1984, 1985) showed that the most posterior tail muscle cells arise from lineages that do not produce muscle autonomously, and that evidently require interblastomere interaction for muscle specification to occur. Thus, since the same muscle genes are activated in autonomously and nonautonomously differentiating muscle lineages, it seems reasonable that in this case as well *active* and *inactive* forms of the same regulatory factors may be present in different blastomeres. Thus to obtain the most posterior muscle cells specific signal transducing events might normally be utilized to activate these regulatory factors, whereas inductive processes are not needed in the major autonomously differentiating muscle lineages.

iii. *Amphibian embryos*

Cleavage stage interblastomere interactions, e.g. those that result in specification of mesodermal lineages, have been more intensively studied in amphibians than in any other forms. The inducing ligands are apparently members of families of evolutionarily related growth factor proteins (e.g. Weeks & Melton, 1987; Kimelman & Kirschner, 1987; Slack *et al.* 1987; Rosa *et al.* 1988) that in mammalian systems are likely to function by receptor-mediated signal transduction. This evidence provides a convincing model for the nature of the interblastomere induction mechanism for other deuterostome embryos. Similarly, LiCl treatment also results in respecification in amphibian embryos (e.g. Kao *et al.* 1986). Two interesting contrasts with sea urchin embryos are as follows: (a) The inducing ligands are diffusible in frog eggs, and act over distances that are several large cell diameters; while on the other hand cleavage planes in frog embryos are not invariant, and lineages of sibling embryos are not identical. Instead given *regions* of the cytoplasm tend to be distributed to cells that display given probabilities of generating progeny of certain cell fates (see Dale & Slack, 1987; reviewed by Davidson, 1986, pp. 210–218). These two facts may be related. That is, for a small system with geometrically precise cleavage planes (e.g. the egg of *S. purpuratus* is only 75 μm in diameter, while that of *Xenopus* is 1200 μm), the ligands might have to be tethered to the presenting cell surfaces, while if they can diffuse, the precise cell surface *positions* become less important. (b) The early inductive events in the frog occur during a period of transcriptional silence, while in the sea urchin they occur during a period of great transcriptional activity that probably includes zygotic synthesis of regulatory factors. In each case, however, plastic cell specifications occur during cleavage. Progeny that are fixed in their fates (and that express specific genes required for their differentiated functions) begin to appear within a division or two after transcription resumes, which in *Xenopus* (e.g. Heasman *et al.* 1984, 1985), is in early gastrulation, while in the sea urchin, since there is no interruption of tran-

scription, this occurs much sooner, i.e. as discussed above, in late cleavage. Perhaps in both cases, however, the decrease in plasticity is due in part to positive autoregulation of the regulatory genes by their own products (which of course requires transcription).

iv. *Mammalian embryos*

Postimplantation mammalian development in certain ways resembles regulative development of sea urchin egg fragments or blastomere aggregates, in that cell fate is evidently specified *de novo* with little or no reliance on the original maternal spatial coordinates. Specification of almost all of the *bona fide* embryonic cell types (i.e. those analogous to the definitive cell types of the other embryos discussed) occurs after implantation. Thus it might be predicted that the pluripotentiality of the lineage precursors in the early postimplantation mammalian embryo (Beddington, 1983; Lawson & Pederson, 1987) also implies the global presence of *potentially* lineage-specific regulators. The regional specification of the initial cell types might be mediated by inductive intercellular interactions that alter the state of these regulators from inactive to active.

Perhaps this is indeed the basic deuterostome plan of action. The localized autonomous cell fates occasionally observed, as in sea urchin skeletogenic cells, or the major ascidian muscle-forming cells, are rather to be considered exceptional devices. Their significance for us is in presenting an illustrative contrast, but for the embryo it is perhaps simply that they are one element of a complex of adaptive mechanisms that facilitate the very rapid development of these free-living forms.

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