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# Programmed development in the mouse embryo

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## 1. THE NATURE OF PROGRAMMED DEVELOPMENT

The programmed development of the embryo involves the specification of cells in space and in time. Specification of cells according to their location is evidently achieved by a process of cell interaction and the recognition of relative cell position. Some positional cues function by setting up within a single cell asymmetries of organization that result in intercellular differences after mitotic division. Others operate within a founder population of homogeneous precursor cells, generating variety via inductive or graded diffusional signals of various sorts (reviewed for various species by Slack, 1983; Johnson & Pratt, 1983). The molecular nature of the cues that convey positional information is known for few, if any, developmental systems, but the available evidence suggests that the cues themselves are unlikely to be highly specific informational molecules. Rather, specificity is achieved through the interaction of positional signals with target cells 'primed' to respond to them. The 'priming' allows the target cells a limited period of developmental competence during which the appropriate differentiative response may be elicited, and thus it reflects the expression of the temporal part of the developmental programme. At its simplest this expression of the developmental programme could be mediated by some stage-specific 'selector'

gene product(s) resulting from the previous set of developmental decisions. Since a cell at any point in development has the capacity to respond in only a limited number of ways (probably only two, Gardner, 1982; Beddington, 1983), we can envisage the temporal programme of development as the sequential presentation of a series of options, each of which at any given time reflects the developmental history of the cell, that is, its lineage. Thus the competence of a cell is defined by its history, and it is this molecular inheritance that provides the substrate upon which cell interactions can operate to select one particular developmental path. The option made available at any point in development, and the selection process that specifies which of the alternatives on offer shall be taken, must be explained ultimately in terms of the availability, selection and stabilization of subsets of genes.

Developmental studies indicate that a number of operational differences in chromatin organization may need to be explained in molecular terms and should certainly be distinguished conceptually. The extreme states consist of (i) inactive and non-activatable chromatin, and (ii) active or activatable chromatin in differentiated cells, in which stable and heritable changes to the chromatin may be discerned. These two states have been distinguished clearly for a number of genes by their differential sensitivity (both within the genes themselves and in their flanking sequences) to DNase 1 and S1 Nuclease (Weintraub & Groudine, 1976; Miller *et al.* 1978; Garel & Axel, 1976; Garel, Zolan & Axel, 1977; Affara, Daubas, Weydert & Gros, 1980; Larsen & Weintraub, 1982; reviewed Elgin, 1981 and Mathis, Ondet & Chambon, 1980) and by their relative methylation levels (reviewed Bird, this volume). Modifications to the nature and composition of associated proteins (Weisbrod & Weintraub, 1979; Levinger & Varshavsky, 1982; Baer & Rhodes, 1983), to the conformational state of the DNA itself (Isenberg, 1979; Allfrey, 1980; Jakobovits, Bratosin & Aloni, 1980), to the actual linear arrangement of the DNA sequence (Storb, Arp & Wilson, 1979; Nasmyth, 1982; Schubach & Groudine, 1984) and an association with the nuclear cage (Gerace, Blum & Blobel, 1978; Cook & Brazell, 1980; Jackson, Eaton, McCready & Cook, 1982) have also been proposed to distinguish these extreme states. However, the transition from non-activatable to active chromatin almost certainly does not occur in a single step, and it is important to determine the state of chromatin when it is (iii) made available as part of the temporal programme of development for selection (or rejection) by positional cues, and (iv) actually selected as a result of the action of positional cues (specification?) prior to being stabilized in a state that ensures heritability (state (ii) above: commitment?). Elegant studies by Weintraub and his colleagues on terminally differentiated cells and cell lines provide an example of the level of sophistication required in such studies (Burch & Weintraub, 1983; Groudine & Weintraub, 1982).

In addition to understanding the nature of chromatin 'in decision', and how this state is either stabilized in an available mode or relaxes back to an inactivated mode, it is also important to determine the ways in which the temporal and

positional cues interact with chromatin. The little evidence so far available suggests that the process of selection may not be quantal, but rather may entail initially reversible and possibly serially cumulative changes involving several levels of signalling both within and outside the chromatin. This conclusion comes not only from the observed lability of developmental decision making in the interval between initial specification and commitment, but also from some evidence that post-transcriptional events may play an important role over this period. In *Dictyostelium*, an early event immediately prior to generation of cell diversity is the aggregation-dependent synthesis of many new species of mRNA. The expression of developmentally significant mRNAs requires cell contact initially but subsequently is maintained by cAMP alone. The cAMP also stabilizes the developmentally regulated mRNAs, and in the absence of continuing cell contact and/or cAMP they are rapidly and selectively destroyed and further synthesis is switched off (Alton & Lodish, 1977; Takemoto, Okamoto & Takeuchi, 1978; Landfear & Lodish, 1980; Chung, Zuker & Lodish, 1981; Gross *et al.* 1981; Mangiarotti, Ceccarelli & Lodish, 1983). Thus, the intracellular response to the initial contact-dependent event must be sustained if development is to proceed. Only subsequently does the cell diversification into stalk and spore cells commence, and here also low-molecular-weight molecules, DIF and $NH_4^+$, act as the signal for diversification. Initially both sets of precursor cells accumulate new mRNAs, but then subsequently subsets are lost differentially (Tsang, Mahbubani & Williams, 1982; Kay, 1983). The impression here is that initially both of the sets of genes available for selection might be expressed, but that positional signals shut down the inappropriate set and stabilize those that are appropriate.

A similar conclusion could be drawn from recent results obtained on *Drosophila* development. Thus, the accumulation of the mRNA product of the homoeotic gene *Antennopedia*, specifying the mesothoracic segment, is restricted to the mesothorax during larval stages. However, at the earlier embryonic stages transcripts are also detected in adjacent non-mesothoracic segments and the segmental restriction occurs with time, is associated with a shift in the subcellular location of transcripts from nucleus to cytoplasm, and possibly also with the expression of other defined homoeotic genes such as *bithorax* and *Ubx* (Levine, Hafen, Garber & Gehring, 1983; Hafen, Levine, Garber & Gehring, 1983; Hafen, Levine & Gehring, 1984). There are several explanations for this observation of which one is an interaction of primary transcripts with positional signalling molecules which restrict the ultimate stabilization of genetic expression to the appropriate segment.

Very early postfertilization development also provides a useful opportunity to determine the influence of post-transcriptional events on development, since transcription at these stages is insignificant and embryogenesis proceeds entirely by modification, turnover and/or selection of mRNA and polypeptides. Thus, specification of the primary axes of development and the early cell lineages

occurs independently of concurrent transcription, and it is presumably the functional segregation of informational molecules that is responsible for the subsequent differential selection of genes in different cells. Regional differences in subsets of mRNA, due to segregation or local differential stability, are found in the sea urchin (Ernst, Hough-Evans, Britten & Davidson, 1980) and *Ascidian* embryos (Jeffery, 1983) and precede identifiable regional differences in transcriptional activity. However, regionalization of mRNA as an early indicator of differentiation is not restricted to early development, witness the segregation of mRNA for flagellin at division in the bacterium *Caulobactor* (Milhausen & Agabian, 1983).

The conclusions that can be drawn from available evidence must be both tentative and speculative. We do not know whether only a limited subset of available stage-specific genes is switched on in any one cell as a result of positional cueing, or whether all available stage-specific genes switch on in every cell, and then positional cues stabilize only a subset in the on position or the complementary subset in the off position. Nor do we know the extent to which transcripts of stage-specific genes interact with positional or lineage-cueing molecules to feedback and regulate chromatin expression during the making of a developmental decision. The important point of distinction to be made, however, is clear. Cells in the process of making a developmental decision may appear very different from cells that have made that decision. The period of developmental decision making may be a period of biochemical ambiguity and it is more difficult to define ambiguous states than quantal events. There are also simple practical problems. Clearly, when studying any developmental system there is a difficulty in capturing a sufficient number of cells in the same developmental state during the transition through specification to commitment. This difficulty will handicap attempts to find explanations at the molecular level. What progress has been made in this direction in the mammal?

## 2. APPROACHES TO STUDYING PROGRAMMED DEVELOPMENT IN MAMMALS

The approaches for studying the molecular basis of programmed development in mammals are similar to those applied to other species, but some of the problems encountered are unique. Thus the numbers of embryos that are readily available is relatively low, the time course of development is relatively slow with long cell cycles and long generation times, and the viviparous nature of development reduces opportunities for recognizing interesting developmental mutants. Those mutants affecting fundamental developmental options will tend to be lost by abortion, and the only mutations to be recognized easily will be those that (i) affect genes expressed relatively late in embryogenesis, (ii) are expressed in non-essential developmental processes (such as, for example, gonad formation and function), or (iii) are expressed in a non-lethal form in the hemizygote. Broadly,

three complementary approaches to the study of mammalian development have been employed, namely: (i) the identification of spontaneous or engineered mutant genes (or gene classes) during embryogenesis from which inferences may be drawn about the time, site and mode of activation of genes crucial to development, (ii) the systematic analysis of developmental cell cycles in relatively homogeneous embryonic cell subpopulations for evidence of changes in DNA, RNA and protein activity around the time developmental decisions are being made, and (iii) the use of cell lines such as embryonal carcinoma cells, myoblasts and erythroblasts as model systems for studying elements of development *in situ*. We will review the results obtained by use of the first two approaches, and will refer to the use of cell lines for supporting evidence.

(i) *Retrospective analysis*

The approach here is to identify a developmentally interesting gene (or gene cluster) and track down its organization and role. The approach relies heavily on serendipity and a sharp eye, and has so far been more informative about the state of commitment in the differentiated cell than about the processes of specification and commitment themselves.

*(a) Endogenous genes*

In mammals no clearly 'homoeotic' genes have been described that lock cells into a developmental pathway normally offered as one alternative in a choice for positional selection (cf. *Drosophila* and *Caenorhabditis*, Lewis, 1978; Bender *et al.* 1983; Greenwald, Sternberg & Horwitz, 1983: but see below) unless the sex-determining role of the Y-chromosome is viewed as 'homoeotic', (see Graves, 1983; Jones, 1983 and later). Some evidence for 'timer' genes in mammals has been reported (Lusis, Chapman, Wangenstein & Paigen, 1983; McLaren & Buehr, 1981). Most genes identified as exerting effects during embryogenesis appear to have diffuse or metabolic effects, and to be involved with housekeeping functions (see Magnuson & Epstein, 1981 for review). The t-locus complex in mice is the most studied system of developmental mutations in the mouse, offering as it does a range of stage- and tissue-specific effects, the genetics of which have been well characterized (reviewed Andrews & Goodfellow, 1982; Magnuson, 1983). Although conclusions about the precise nature and mode of action of the t-complex gene products are at best controversial, the recent identification of primary polypeptide products of t-complex encoded mRNAs and of restriction fragments unique for specific t-haplotypes, promises a resolution of these controversies (Silver, Artzt & Bennett, 1979; Shin, Staunezer, Artzt & Bennett, 1982; Silver, 1982; Silver, Uman, Danska & Garrels, 1983).

In addition to the identification of individual developmental genes, attention has been focussed recently on the role that expression of classes of gene product might have in development. For example, Murphy *et al.* (1983) have reported that 1–3 % of transcripts detected between 6·5 and 10 days of development in

mouse embryos contain a repetitive sequence with some features of a transposable element. The sequence is also present as a free polynucleotide, transcribed by RNA polymerase III. This repetitive sequence was initially recognized among the transcripts in 3T3 cells transformed by SV40, and is also present in other transformed cell lines and in pluripotent embryonal carcinoma cell lines. The frequency of the transcripts containing this sequence declines in embryos from 10 days onwards and also after induction of differentiation in pluripotent cell lines. One transcript containing the repetitive sequence seems to show similarities to a Qa/Tla Class 1 medial histocompatibility antigen (Brickell *et al.* 1983). It is speculated that in this instance the repetitive elements might accompany sequences encoding proteins involved in cell–cell recognition, analogous to those involved in the immune response, and possibly therefore involved in recognition or signalling of position during early embryogenesis. However, until the nature and range of other transcripts bearing the repetitive sequence marker is established, it is premature to ascribe restricted functions to the whole class of mRNAs. There is at present little or no information on when the marked transcripts first appear, and on their cellular and subcellular distribution (Rigby, 1984).

Although this report was the first example of a repetitive element being associated with a particular stage of development in mammalian embryos, a tissue-specific 82-nucleotide element, expression of which is restricted in the adult rat to neural tissue, had been described previously (Sutcliffe, Milner, Bloom & Lerner, 1982). This element can appear either as an intron in the alpha-amanitin-sensitive primary transcripts present in neural tissues, or within two cytoplasmic species, one of 110-nucleotides which is probably not polyadenylated (BC2), the other a 160-nucleotide polyadenylated species (BC1). Both BC1 and BC2 have been shown to be transcribed not by RNA polymerase II but by RNA polymerase III, and are thus insensitive to alpha-amanitin. Only brain nuclei act as *in vitro* templates for the synthesis of this neural identifier sequence, unless nuclei from other tissues are first salt treated to alter the associations with chromosomal proteins when they will also synthesize primary transcripts containing the identifier sequence, but not the smaller, Pol III transcribed species (Sutcliffe, Milner, Gottesfeld & Lerner, 1984). It is proposed that the identifier sequence is necessary for neural-specific gene expression but not in itself sufficient. The activation of non-neural tissue chromatin following salt washing suggests that there is normally a negative control exerted by specific repressors, whilst a derepression in neural tissue allows selection of this subset of genes marked with the identifier sequence.

A third category of identifier, repetitive sequence in mammals has been defined by a subclone of the BKM probe, originally derived from satellite DNA of a snake, the banded krait, and shown to hybridize in a sex-specific manner within murine chromosomes thereby identifying the male-determining region of the Y-chromosome (Jones, 1983; Singh, Phillips & Jones, 1984). The probe has

been used to search for evidence of transcription of the repetitive sequence within a poly(A)+ RNA molecule. Various-sized transcripts associated with the repetitive sequence were detected in adult male liver and kidney, but not in female tissues, embryonic male liver, or tissue from females treated with androgens. The expression of the sequence appears to be developmentally regulated and sex specific.

Thus, three systems of identifier sequences have now been described in mammals, one evidently characteristic of a transitional and pluripotential lineage, one of a terminal cell lineage and one that is sex specific and developmentally regulated. In each case there is a proposal that the sequence marks a broad class of transcripts, as well as being present in two cases as part of a Pol III transcribed polynucleotide sequence. There is a suggestion that transcribed identifier sequences might also exist that are characteristic of liver and kidney (Sutcliffe *et al.* 1984). Identifier sequences that are repeated extensively throughout the genome have not only been described in mammals. Analogous elements have been reported in other systems such as *Dictyostelium* (Chung *et al.* 1983; Zucker, Cappelo, Chisholm & Lodish, 1983), *Drosophila* (Tchurikov, Naumova, Zelentsova & Georgier, 1982), *Xenopus* (Anderson *et al.* 1982) and sea urchins in which developmental regulation of their expression occurs (Grosschedl & Birnstiel, 1980; Posakony, Flytzanis, Britten & Davidson, 1983). In *Drosophila*, a transcribed 180 base pair sequence has been found associated with genes specifying aspects of segmental information (McGinnis *et al.*, 1984). A similar sequence is also present in limited copy number in the DNA of a number of species including *Xenopus* and man (Shepherd *et al.*, 1984), and encodes for a protein domain similar to that known to bind to DNA in prokaryotes (Laughon & Scott, 1984). In *Dictyostelium* these repeats also show properties associated with transposable elements. The marking of a class of developmental mRNAs in this way could provide a mechanism for making available coordinately a whole group of genes for expression in response to a given signal, whether that signal be part of the temporal or spatial programme (Davidson, Jacobs & Britten, 1983). It will be of interest to couple stage-specific identifier sequences to alien genes and to observe whether the timing and/or location of their expression is brought under developmental regulation (see later). It will also be of interest to unravel the extent to which different cells within a lineage express different subsets of similarly marked transcripts, and whether any such imbalance found can be influenced by changing positional cues. Evidence for an interaction between an environmental signal for development and the expression and stabilization of a marked set of mRNAs has already been obtained in *Dictyostelium* (Zucker *et al.* 1983), and the repetitive sequence in the mouse pluripotent cells has been shown to bind SV40 large T antigen. Such binding has been implicated in the regulation of expression of a number of cellular genes during transformation, and is thought to represent viral mimicry of a natural cell regulatory protein (Dynan & Tijian, 1983; North, 1984) that may represent one example of a general class of such proteins (Scholer & Gruss, 1984).

*(b) Introduced genes*

Indirect evidence consistent with the involvement of flanking (or identifier) sequences in the regulation of developmental gene expression in mammals has come from an altogether different approach to the analysis of developmental programming, in which exogenous genetic material is introduced into the mouse genome. With this approach 'strains' of mice carrying the novel sequences can be produced, and the inserted material transmitted to progeny via the germ line (Jaenisch *et al.* 1983; Wagner, Corarrubias, Stewart & Mintz, 1983). Embryos can then be probed either for the activity of the introduced genetic material itself or for any disruptive effect it may have on endogenous gene activity. The two most successful techniques used are retroviral infection at various stages of development (Jaenisch, 1983) and injection of cloned material directly into the male pronucleus of fertilized single cell embryos (Costantini & Lacey, 1981; Wagner, Stewart & Mintz, 1981; Brinster *et al.* 1981; Gordon & Ruddle, 1981). With both these techniques there is little evidence (from *in situ* hybridization and analysis of flanking sequences) of systematic insertion at specific sites. However, it is not clear that insertion is entirely random, 'hotspots' for insertion (such as regions of DNase hypersensitivity) that may vary during development perhaps being favoured loci (e.g. Storb *et al.* 1979; Nasmyth, 1982; Schubach & Groudine, 1984).

Gene injection has the distinct advantage that many different cloned sequences of DNA can be introduced, the composition and size of the constructs can be varied, and, since integration seems to occur rapidly, the embryo that develops is less likely to be a genetic mosaic. However, this approach requires considerable experimental dexterity, must be done earlier than retroviral infection and yields a lower rate of successful integration into host DNA up to a maximum of 40 %. Integration of the foreign DNA is not chromosome specific, and the number of integrated copies can vary dramatically from one to hundreds, usually but not invariably, found arranged head to tail in a tandem array at a single insertion site. The level of expression of such exogenous DNA once integrated into the genome varies. Initial experiments with beta-globin (Costantini & Lacey, 1981) and thymidine kinase genes (Wagner *et al.* 1981) showed low, variable and temporally inappropriate levels of expression in a range of cell types, and it is presumed that this expression was regulated via transcriptional activity in adjacent host DNA. Even when genes were fused to strong promoters developmental control was not achieved, although when the genes did become active they were susceptible to a measure of control via their own promoter (i.e. thymidine kinase or rat growth hormone gene fused to the metallothionein promoter: Palmiter *et al.* 1982*a*; Palmiter, Chen & Brinster, 1982*b*). Only when rearranged immunoglobulin heavy chain genes, containing their own enhancer (Mercola, Wang, Olsen & Calame, 1983; Gillies, Morrison, Oi & Tonegawa, 1983; Banerji, Olson & Schaffner, 1983) and coupled to a metallothionein

promoter, were used, was a measure of developmental control achieved (Brinster *et al.* 1983). These results suggest that (i) the chromosomal position of such exogenous genes influences their expression primarily negatively, (ii) that endogenous control programmes can take foreign genes under their control, and (iii) that possibly the presence of enhancer sequences might confer a measure of developmental control to the foreign gene, although this conclusion needs further support.

Comparable experiments in *Drosophila*, in which a construct of a specific cloned gene with limited flanking sequences linked to a transposable P-element has been used to introduce the exogenous genetic material (O'Hare & Rubin, 1983), have *not* revealed evidence of site dependence in regulated developmental expression, although some minor quantitative effects of position are evident (Scholnick, Morgan & Hirsh, 1983; Spradling & Rubin, 1983; Goldberg, Posakony & Maniatis, 1983). The differences from the results with mice might reflect the existence in *Drosophila* of a less-extended cis-controlling sequence, the P-element-directed insertion of the gene into particular sites that do not override or inhibit developmental regulatory sequences, a relatively low incidence of such inhibitory or non-permissive chromatin in the recipient, and/or differences in the numbers of mutants produced and the relative ease of their detection and analysis.

Retroviral infection has technical advantages over gene injection; higher efficiency of integration can be achieved and genes can be introduced at both early and late stages of development. Murine leukaemia viruses (MuLV) carry their genetic information as single-stranded RNA molecules and infection involves the synthesis and chromosomal integration of a double-stranded DNA provirus. It is assumed that historically endogenous retroviral-like sequences present within the genome arrived by a similar route. Probes corresponding to the proviral sequence are easy to make and detect, as sensitive methods for determining the expression of the retroviral products are available. Therefore it is possible to monitor expression and modification of this exogenous DNA. Retroviral infection of preimplantation embryos is followed by rapid integration into the host DNA. Before integration, expression of the DNA is possible, but following integration expression ceases (Harbers, Jähner & Jaenisch, 1981*a*; Stewart, Stuhlmann, Jähner & Jaenisch, 1982). Moreover, transfection of the integrated DNA into cells permissive for expression of the introduced genes does not result in gene expression. Thus, the gene has become effectively inactivated. This loss of activity persists during embryonic differentiation; only subsequently and in some embryos are the introduced genes expressed at all, and then only in a restricted number of tissue types, at a time and to a level characteristic of the individual mouse. This characteristic pattern of expression is then generally stable through the germ line (Jähner & Jaenisch, 1980; Jaenisch, Fan & Croker, 1975; Jaenisch *et al.* 1981; Harbers *et al.* 1981*a*). These results argue that the introduced DNA is modified at or around the time of integration into the host

DNA and only comes under developmental control by its chance proximity to a developmentally regulated sequence. One modification that certainly does occur at some point after the integration process is methylation of certain cytosine residues (Stuhlmann, Jähner & Jaenisch, 1981; Jähner *et al.* 1982). Moreover, carefully controlled experiments, involving either methylase inhibitors or methylases from *E. coli* and rat liver, have demonstrated that expression of a proviral sequence is dependent upon, and inversely proportional to, its state of methylation (Harbers *et al.* 1981*b*). However, this observation does not mean that *in situ* methylation is the primary mechanism for inactivation, indeed there is evidence against this possibility (Gautsch & Wilson, 1983; Niwa, Yokota, Ishida & Sugahara, 1983). Experiments in which undifferentiated embryonal carcinoma cells were infected with retroviruses also indicated the presence of *de novo* methylation associated with suppression of activity when the cells were induced to differentiate (Stewart *et al.* 1982). In sharp contrast to these results, it was found that neither the suppression of proviral activity, nor the associated *de novo* methylation of the proviral sequences, occur when postimplantation embryos (Jähner *et al.* 1982) or differentiated embryonal carcinoma cells (Stewart *et al.* 1982) are infected. This implies that the capacity to methylate these sequences *de novo* is stage specific.

More limited data about regulation in development have come from infecting early embryos and embryonal cell lines with the DNA papovaviruses (e.g. polyoma – a murine virus for which mouse cell lines are permissive and SV40 – a monkey virus which can show only early viral expression in mouse cell lines and not usually late expression or viral replication). Infection of early embryos can result in persistence of intracellular viral particles that in some cases has been shown to be due to an early integration event. However, no evidence of viral function has ever been detected in the pluripotent cells of the embryo (morula, ICM, embryonic ectoderm). Only in trophectoderm or later differentiated cell types can evidence for viral proteins (and, in the case of polyoma virus, replication) be found (reviewed by Kelly & Condamine, 1982). Similarly pluripotent embryonal carcinoma cells may be infected by papovaviruses but are not permissive for expression, whilst the differentiated derivatives of these cells do permit expression and, in the case of polyoma virus, replication (Swartzendruber & Lehman, 1975; Kelly & Condamine, 1982). The mechanism by which expression is inhibited is the subject of some controversy (reviewed by Levine, 1982; Kelly & Condamine, 1982). There is evidence to suggest that transcription of viral mRNA may be occurring but that pluripotent cells are unable to splice or stabilize the transcripts. There is also evidence for defects in transcription. Of interest to the current discussion is the production of a number of mutant polyoma viruses all of which are characterized by a change in a non-coding portion of the genome in the region of the enhancer sequence and elsewhere. Each variant shows a capacity to infect, and replicate in, some, but not all, embryonal carcinoma cell lines. The fact that modifications in the structure of the

enhancer region influence the capacity of the viruses to be regulated differentially in host cells, is presumed to reflect an underlying control mechanism the action of which is restricted to specific developmental stages (reviewed by Levine, 1982; see also Linney *et al.* 1984). Thus, three independent approaches, in which exogenous DNA has been integrated into host chromatin, provide evidence that stage-specific sequences which may be enhancer like might provide an important control component in developmental programming.

The approaches to analysing the molecular basis of programmed development in mammals reviewed above have, by their design, involved mainly an analysis of the *outcome* of developmental decision making, rather than the *process* itself. They must, therefore, be viewed cautiously, as a distant view can be a distorted one. However, these 'retrospective' approaches have produced evidence consistent with the view that in mammals there may be a role for stage-specific flanking sequences in the interaction with positional and temporal cues, and that one feature of early development is a generalized 'shut down' of chromatin, later relieved only selectively and under developmental control. These returns may seem meagre considering the effort involved. However, it is clear that despite the limitations and technical difficulties involved, these approaches are already providing recombinant DNA probes with which the DNA and mRNA of cells actually engaged in making developmental decisions may be probed directly. The problem that confronts the application of these probes in mammals is one common to most other systems. Developmental decisions are made in small groups of cells or even in single cells, and the technology required for analysis must therefore be made correspondingly sensitive. Moreover, intelligent use of the probes requires a detailed understanding of the temporal and spatial characteristics of specification, commitment and differentiation at the cellular level. So the complementary approach to the retrospective study of developmental programmes is to analyse cells at the time of their decision making for evidence of changes in the interactions of DNA, RNA and protein and in the nature of the relationships of these activities to the cell properties as a whole. In this respect early mammalian embryos have distinct advantages, as they are robust and readily manipulated, and their long cell cycles permit analysis of cell-cycle related, developmental decisions. It is for this reason that the most detailed studies on mammals have been undertaken on preimplantation embryos.

(ii) *Programmes and patterns in preimplantation development*

The first eight cell cycles of mouse development are characterized by long cell cycles, no net growth and by the generation of two committed cell subpopulations (the inner cell mass or ICM, and trophectoderm, Fig. 1).

Cell diversity arises by a continuing series of cell interactions that influence (i) the generation and orientation of an axis of polarity in individual constituent blastomeres, (ii) the orientation of cleavage planes in polarized blastomeres thereby determining whether divisions are differentiative or conservative, and

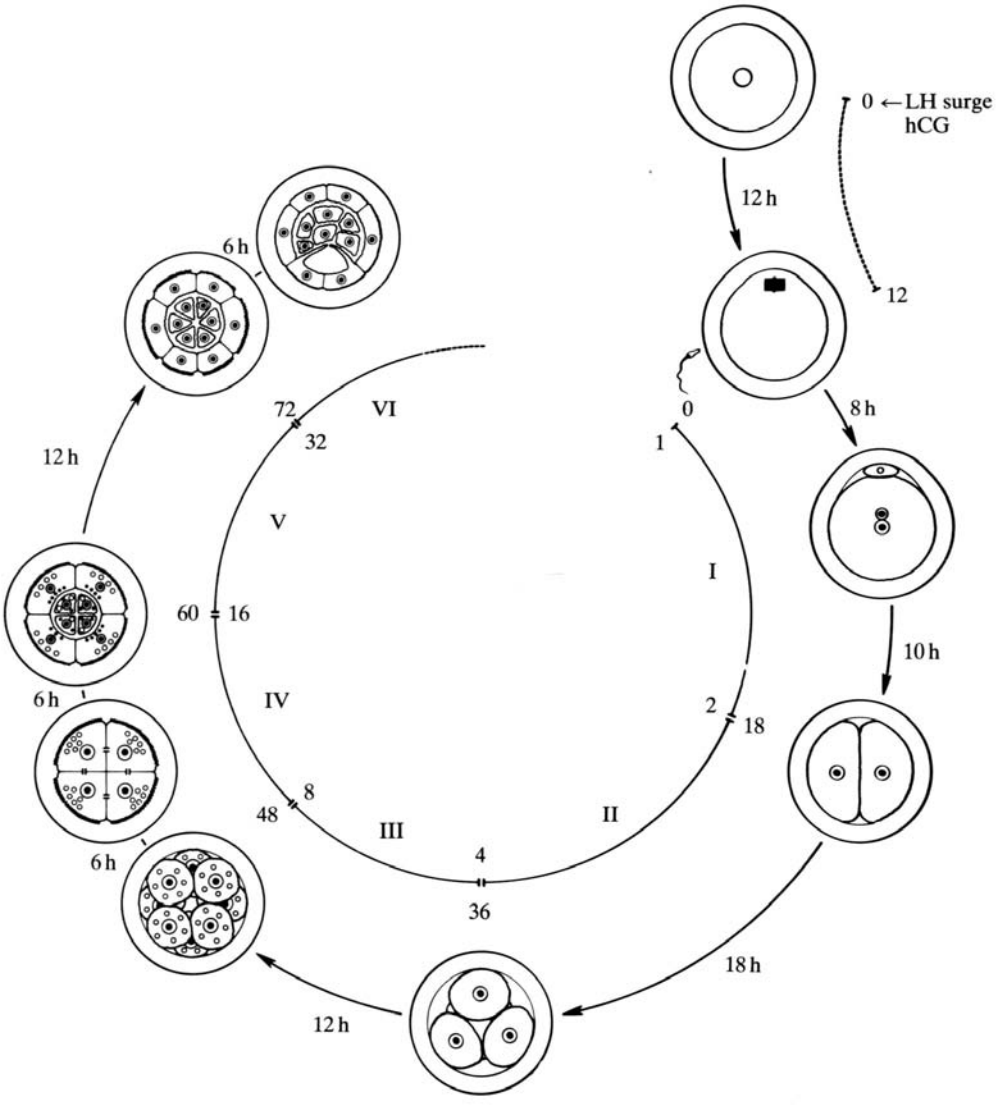


Fig. 1. Development of the mouse blastocyst. Development is initiated with the activation of the preovulatory follicle by endogenous luteinizing hormone (LH) or exogenous human chorionic gonadotrophin (hCG). The germinal vesicle, containing chromosomes in first meiotic prophase, breaks down and meiosis proceeds to second meiotic metaphase over a 12 h period. During this period the egg is ovulated. Insemination at 0 h leads to development through the six developmental cell cycles indicated internally in Roman numerals, cleavage occurring at approximately 18, 36, 48, 60 and 72 h post insemination. Polarization of blastomeres, intercellular flattening and junction formation occur in the IVth cell cycle and the blastocyst forms in the VIth cell cycle, the blastocoel, inner cell mass and trophectoderm being visible.

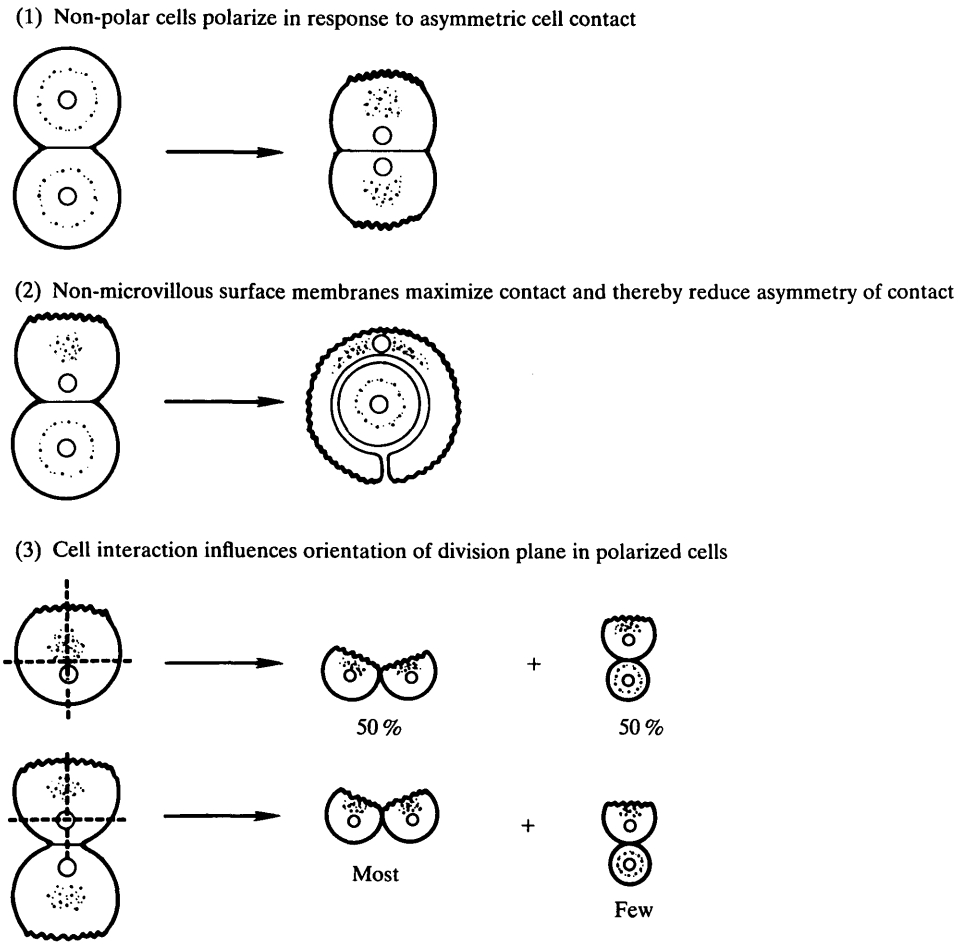


Fig. 2. Schematic summary of principal cellular mechanisms involved in generating cell diversity in the blastocyst. (1) Non-polar cells at the 8-, 16- and 32-cell stages polarize in response to asymmetric cell contact, their nuclei migrate basally, endosomes aggregate apically and a surface pole develops opposite to the point of contact. (2) Polarization is inhibited if the non-polar cell (in this case a 1/16 cell) is totally surrounded either by many cells or, as shown here, by a single polar cell enveloping it. In the absence of total cell contact, polarity is generated as shown in (1). (3) The proportion of polar cells dividing conservatively or differentiatively varies depending upon the contact patterns, as summarized here for polar 1/16 cells. (See Johnson, 1985 for detailed discussion.)

(iii) the extent to which cells sort to the inside or outside of a cell cluster via their differential adhesive properties. These three mechanisms provide the means by which relative cell position in the morula is converted to committed cell lineage in the blastocyst (Fig. 2; reviewed in detail Johnson, 1985). The process of positional recognition probably commences as early as the second cell cycle, is labile during the third, fourth, fifth and probably sixth cell cycles and commitment has probably occurred by the eighth cell cycle at the latest.

*(a) The molecular time table of early development*

The molecular basis for the events of early embryogenesis has been studied in some detail especially for the very earliest stages. It is convenient to view the initiation of development as the resumption of the arrested cell cycle of the intrafollicular primary oocyte, which has rested in prophase of the first meiotic division since foetal life (the Germinal Vesicle (GV) stage; Fig. 1). The nuclear changes during the transition of the primary oocyte to the ovulated secondary oocyte arrested in metaphase of the second meiotic division take about 12 h in the mouse, and are accompanied by numerous qualitative and quantitative changes in protein synthetic profile (mouse: Cascio & Wassarman, 1982; sheep: Warnes, Moor & Johnson, 1977; pig: McGaughey & Van Blerkom, 1977; Richter & McGaughey, 1982), by major and dynamic shifts in the distribution of cell organelles such as mitochondria, lysosomes and cortical granules (Zamboni, Thompson & Moore-Smith, 1972; Ezzell & Szego, 1979; Van Blerkom & Runner, 1984; Kruip, Cran, Van Beneden & Dieleman, 1983), development of perinuclear microtubule organizing centres (Szollosi, Calarco & Donahue, 1972), maturation of cytoplasmic factors that subsequently influence pronuclear development (Thibault, 1977; Usui & Yanagimachi, 1976) and reorganization of the cell surface topography and its underlying subcortical matrix (Nicosia, Wolf & Inoue, 1976; McGaughey & Van Blerkom, 1977; Van Blerkom & McGaughey, 1978*a,b*). With fertilization, release of cortical granules and changes in cell membrane properties occur, the second polar body is extruded, the sperm nucleus is incorporated and its chromatin decondenses, pronuclei form, migrate centrally, replicate their DNA, and only at the first metaphase (16–19 h postfertilization in the mouse) do the two haploid sets of chromosomes come together for the first time on the spindle (summarized in Maro, Johnson, Pickering & Flach, 1984). The morphological changes of this first cell cycle are also accompanied by quantitative and qualitative changes in protein synthetic profile (Van Blerkom & Brockway, 1975; Levinson, Goodfellow, Vadeboncoeur & McDevitt, 1978; Cullen, Emigholz & Monahan, 1980; Van Blerkom, 1980, 1981*a,b*; Cascio & Wassarman, 1982; Pratt, Bolton & Gudgeon, 1983; Howlett & Bolton, 1985). The highly characteristic change with time in the profile of proteins synthesized throughout this early phase of development from germinal vesicle breakdown (GVBD) to the 2-cell stage represents the expression of a developmental programme that appears to function entirely independently of proximate transcription, using transcripts synthesized during oocyte growth prior to GVBD (Brower, Gizang, Boreen & Schultz, 1981).

Direct evidence for transcriptional activity at these stages is limited to detection of very low levels of incorporation of radiolabelled adenosine into an unidentified species of heterodisperse high molecular weight RNA (Clegg & Piko, 1982, 1983*a,b*) and to the detection of nascent fibrils on chromatin of pig oocytes (McGaughey, 1983). Many other failures to detect synthesis of RNA have been

reported (e.g. Rodman & Bachvarova, 1976; Wassarman & Letourneau, 1976; Moore & Lintern-Moore, 1978; Young, Sweeney & Bedford, 1978; Moore, 1975; Mintz, 1964). Moreover, chemical enucleation, and physical removal of pronuclei, do not appear to affect the tempo or character of polypeptide changes over this period (Braude, 1979*a,b*; Braude, Pelham, Flach & Lobatto, 1979; Van Blerkom, 1981*a*; Cascio & Wassarman, 1982; Pratt *et al.* 1983; Petzoldt, Hoppe & Illmensee, 1980; Flach *et al.* 1982; Bolton, Oades & Johnson, 1984; Howlett & Bolton, 1985). Finally, when *in vitro* translates of mRNA extracted from oocytes and 1-cell eggs are compared, it is found that the changing protein synthetic profiles cannot be explained by changing mRNA populations (Braude *et al.* 1979; Van Blerkom, 1981*a*; Cascio & Wassarman, 1982; Pratt *et al.* 1983; Howlett & Bolton, 1985).

The complexity of the *in vitro* biosynthetic pattern is much less than that of the *in situ* patterns at different stages, and it is clear from peptide mapping of polypeptides synthesized *in situ* that a limited number of families of related polypeptides exist, and that most of the variation in polypeptide synthetic profile with time can be accounted for by a temporal programme of post-translational modification or changing stability of polypeptides (Van Blerkom, 1981*b*; Cascio & Wassarman, 1982). This conclusion is reinforced by the results of pulse-labelling experiments in which label can be chased from one group of polypeptides to others in a characteristic and time-dependent manner (Pratt *et al.* 1983; Howlett & Bolton, 1985), and by analysis of the changing temporal pattern of peptide glycosylation and phosphorylation (Van Blerkom, 1981*b*; Pratt *et al.* 1983; Schultz, Montgomery & Belanoff, 1983). Evidence is available from other species that selective modification and/or stability of polypeptides might show temporal variation in early development e.g. the sea urchin (Evans *et al.* 1983) and *Xenopus* (Maller, Wu & Gerhart, 1977).

Whilst the bulk of polypeptide synthetic changes observed between resumption of meiosis and division to two cells appear to involve post-translational modification, a few changes are more easily explained on the basis of time-dependent changes in the rate of utilization of a specific subset of mRNAs (Braude *et al.* 1979; Van Blerkom, 1981*a*; Cascio & Wassarman, 1982; Howlett & Bolton, 1985). The changing translational rates associated with this subset of mRNA cannot be explained easily by general changes in the 5′ capped or 3′ tailed status of the messages (Schultz, Clough & Johnson, 1980; Johnson, 1981; Bolton, 1984), and is unlikely to be explained by temporal variation in the capacity to process primary transcripts (Bachvarova, Kaplan & Jelinek, 1983) although this remains to be proven directly. Evidence for selective use of subsets of mRNA has also been reported for the early development of *Spisula* (Rosenthal, Hunt & Ruderman, 1980; Ruderman *et al.* 1983), and the sea urchin (Raff, 1983), although the precise regulatory mechanisms used in each case are different. Qualitative changes in the population of proteins synthesized could also be achieved if the rate of protein synthesis varied markedly during development to

2 cells, since at higher synthetic rates less-efficient messages would be more likely to be recruited. However, unlike the situation in the sea urchin, fertilization in the mouse is not marked by a major shift in biosynthetic activity which increases at the 8- to 16-cell stage (Brinster, Wiebold & Brunner, 1976). Calculations of the translational efficiency of mRNAs for histone and actin suggest values of about 0·09 polypeptides/mRNA/minute in the unfertilized egg, which is some 1/20th that reported for the blastocyst and for somatic cells (Giebelhaus, Weitlauf & Schultz, 1985). There is other evidence to suggest that protein synthesis is relatively inefficient in the egg and that the functional translational apparatus might be limiting. Thus, Ebert & Brinster (1983) found evidence of competition between exogenous rabbit beta-globin mRNA injected into mouse eggs and endogenous mRNAs, suggesting a limited biosynthetic capacity in eggs. Moreover, Bachvarova & De Leon (1977) provided evidence that the bulk of ribosomes in the ovulated egg are not active in protein synthesis and cannot be recruited into synthetic activity *in vitro*. The egg appears to translate inefficiently, and not to show appreciable changes in translational rate until later in cleavage by which time much new ribosomal synthesis has occurred (LaMarca & Wassarman, 1979; Clegg & Piko, 1977; Piko & Clegg, 1982).

Most of the proteins synthesized during these early phases of development up to the first cleavage division remain detectable qualitatively until the morula stage (Pratt *et al.* 1983) and quantitative studies also suggest that most proteins have a long half life (Brinster *et al.* 1976). However, whilst the proteins may persist far into development, the messages that coded for them do not. There is a physical loss of maternally derived mRNA from the embryo so that by the late 2-cell stage most, if not all, maternal mRNA has gone. Most of this loss occurs during the 2-cell stage as evidenced by the disappearance of alpha-aminitin-insensitive transcripts (Flach *et al.* 1982; Bolton *et al.* 1984) and labelled maternal RNA (Bachvarova & De Leon, 1980), a dip in total or polyadenylated RNA (Piko & Clegg, 1982; Clegg & Piko, 1983*a,b*), and the decline of maternal transcripts for histone H3 and actin (Giebelhaus *et al.* 1983). However, an early loss at the 1-cell stage of maternal transcripts related to intracisternal large A-type particles has been reported recently (Piko, Hammons & Taylor, 1984), suggesting the existence of at least two phases of destruction. The maternal mRNA is replaced during the 2-cell stage with embryonic mRNA, as revealed by major changes in the *in situ* and *in vitro* translation patterns (Van Blerkom & Brockway, 1975; Bolton *et al.* 1984), an acquired sensitivity to alpha-amanitin (Moore, 1975; Levey & Brinster, 1978; Flach *et al.* 1982; Bolton *et al.* 1984), the incorporation of radiolabelled precursors internally into mRNA species (Young *et al.* 1978; Levey, Stull & Brinster, 1978), and the appearance of new transcripts coding for products identifiably derived from the newly constituted embryonic genome (Sawicki, Magnuson & Epstein, 1981), including transcripts for a subtype of H3 histone distinguishable from those synthesized in the oocyte (Giebelhaus & Marzluff, personal communication).

The embryonic gene activity has been timed with some precision and shown to occur in two bursts, the first immediately before and the second immediately after the period of DNA replication (Flach *et al.* 1982; Bolton *et al.* 1984). The mRNA activity appearing during G1 results in synthesis of a small group of polypeptides characterized as being very similar to the heat-shock proteins hsp 68 and 70 by their mobility and peptide digestion patterns (Bensaude, Babinet, Morange & Jacob, 1983). Early in G2, large numbers of embryonic transcripts appear, some coding for proteins similar to those synthesized on maternally derived transcripts but many others coding for new developmental proteins (Van Blerkom & Brockway, 1975; Sawicki *et al.* 1981; Giebelhaus, Heikkila & Schultz, 1983; Bolton *et al.* 1984). The production of new transcripts in G1 and early in G2 of the second developmental cell cycle is linked tightly to the translation of detectable polypeptide products which appear within 1 to 3 h (Flach *et al.* 1982; Bolton *et al.* 1984). However, quite remarkably, although transcriptional activity continues through the prolonged G2 of the second half of the 2-cell stage and on into later cleavage stages, its inhibition from the late 2-cell stage with alpha-amanitin is nonetheless compatible with many developmental events up to the late 8-cell stage including events concerned with the cell-contact-induced polarization of blastomeres (Johnson & Pratt, 1983 and unpublished), cell flattening at compaction and the development of junctional communication between blastomeres (McLachlin, Caveney & Kidder, 1983). Thus, a short burst of embryonic mRNA appears to supply most of the embryo's needs for the ensuing two cell cycles. The clear implication is that much of the early process of cell interaction and positional recognition that leads to cell diversification can also be regulated at a post-transcriptional level. Indeed, although the molecular events underlying these cell cycles have been studied much less intensively than those of earlier stages, evidence of stage-specific post-translational changes such as phosphorylation has been reported (Lopo & Calarco, 1982). Molecular analysis of subsequent development to the blastocyst remains at a relatively preliminary stage of molecular analysis compared with earlier stages. However, certain tentative conclusions can be drawn. First, during this period the rates of transcription and translation start to rise rapidly and the rates of mRNA and protein turnover change (Brinster *et al.* 1976; Braude, 1979*a*; Kidder & Pedersen, 1982). Second there is evidence that the two different cell subpopulations synthesize different proteins from the 32-cell stage onwards (Van Blerkom, Barton & Johnson, 1976; Handyside & Johnson, 1978). This probably reflects differential production or stability (rather than differential use) of mRNA in at least the later stages prior to commitment (Johnson, 1979).

In many mammals embryonic growth and differentiation arrest at the blastocyst stage, just prior to implantation. This phenomenon, termed delayed implantation, occurs when biochemical and/or morphophysiological conditions in the uterine lumen are inconsistent with implantation (Daniel, 1970). Such a reproductive strategy has evolved to reduce the potential for embryonic wastage

in the presence of adverse uterine conditions, to ensure birth during the appropriate season and in some species, such as the mouse, to maintain a nearly constant state of pregnancy.

The molecular and cellular processes associated with delay of implantation have been studied most extensively in the laboratory mouse, where delay occurs naturally if a mouse is pregnant while nursing a previous litter, or if ovariectomy is performed during the early phases of the preimplantation period (Van Blerkom, Chavez & Bell, 1979). The onset of the delay state is accompanied by a rapid and progressive decline in rates of metabolism and synthesis of protein and RNA and is followed within approximately 24 h by a cessation of DNA synthesis and mitosis in most of the cells of the blastocyst. RNA synthesis during developmental arrest appears to involve mostly turnover activity, ribosomal and transfer RNA being the predominant species transcribed (Chavez & Van Blerkom, 1979). Fine structural changes parallel metabolic and synthetic alterations and include disassembly of polysomes into free ribosomes and changes in composition, appearance and distribution of cytoplasmic components such as mitochondria, lipid bodies and cytofilaments (Van Blerkom *et al.* 1979). Depending upon the species, this developmental quiescence may persist from days to months (Daniel, 1970).

Activation from the delayed state is rapid and involves a restoration of the molecular and cellular conditions that existed prior to the onset of the developmental arrest. Activation appears to include a cascade of molecular, cellular and metabolic changes initiated by oestrogen and resulting in the first signs of implantation within 24 h. While it is clear that extraembryonic (extragenomic) signals are responsible for the initiation, maintenance and termination of the delayed state, the biochemical nature of these signals remains obscure. Factors involved in the induction and termination of developmental arrest may be highly specific uterine macromolecules (Weitlauf, 1978), or relatively simple, nonspecific signals or conditions such as qualitative or quantitative changes in intrauterine availability of amino acids (Van Blerkom *et al.* 1979), metabolic substrates (Wordinger & Brinster, 1976) or ions (Van Blerkom *et al.* 1979; Van Winkle, 1977). Regardless of the nature of these signal(s), the effect on the embryo is pleiotropic and on development is dramatic.

The sequence or cascade of molecular and cellular events that occur during blastocyst activation represent the expression of a resumed developmental programme that conveys to the embryo the ability to attach to and subsequently to implant into the uterine epithelium. Unfortunately, little is known about the molecular nature of this phase of embryo activation. Because the activation of the developmental programme leading to implantation is so rapid, the possibility exists that information required for such a programme is provided but not expressed during the protracted period of quiescence. If it is assumed that differential protein expression is one aspect of such a programme, analysis of protein synthesis should provide clues relevant to the nature of the activation programme. One-dimensional electrophoretic separations of proteins synthesized

before, during and after termination of delay reveal quantitatively and qualitatively similar patterns (Van Blerkom & Brockway, 1975; Chavez & Van Blerkom, 1979). This observation is confirmed by high resolution, two-dimensional separations where less than ten polypeptides could be considered to be 'activation specific' (Van Blerkom *et al.* 1979). There is no evidence at present to suggest that activation-specific proteins are translated from mRNAs synthesized but not expressed during delay of implantation. However, progressive changes in the glycoprotein nature of the cell surface do occur during activation as demonstrated by means of lectin-binding activity and cytochemistry (Chavez & Enders, 1981, 1982). Moreover, preliminary findings demonstrate that activation from delay in the mouse is accompanied by specific changes in patterns of protein phosphorylation (Van Blerkom, 1984*b*). Presumably, both protein phosphorylation and glycosylation are an integral part of the programme of activation from delay. A programme could rapidly 'turn off' and 'turn on' essential cellular activities critical to the progression of development as the blastocyst approaches and prepares for implantation. However, this notion remains highly speculative because the extent to which the activation programme involves post-translational control rather than regulation at the transcriptional or post-transcriptional levels requires more definitive experimental information (Giebelhaus *et al.*, 1985).

*(b) Control in the maternal programmes for early development*

The timetable for early development involves a minimum of three programmes: the oocyte programme commencing with reactivation of the oocyte and breakdown of the germinal vesicle, an activation programme commencing with fertilization, and an embryonic programme commencing with the expression of the embryo's own genes. Recent evidence suggests that these programmes may be preceded by an oocyte growth programme (Canipari, Palombi, Riminucci & Mangia, 1984). How is each of these programmes regulated? To what extent are they autonomous? Are they truly discrete and sequential or does each underlie and run on into the ensuing programme?

*The oocyte programme* is activated via the effect of luteinizing hormone on the hormonally primed granulosa cells of the cumulus mass. The precise nature of the signal transmitted to the oocyte is unclear, but it is possible that an early event is a reduction in cAMP levels (Schultz & Wassarman, 1977) leading to changes in phosphorylation of various phosphoproteins (Schultz *et al.* 1983). Germinal vesicle breakdown (GVBD) then follows, a sequence analogous to that reported for *Xenopus* (Nielson, Thomas & Maller, 1982). GVBD releases proteins previously sequestered in the nucleus, including a $28\times10^3$ relative molecular mass phosphoprotein, enriched 1000-fold in the GV, the synthesis and phosphorylation of which terminates at GVBD (Wassarman, Schultz & Letourneau, 1978). It has been proposed (Van Blerkom & Runner, 1984) that this release of sequestered proteins initiates a cascade reaction of morphological and

molecular changes including striking 'pulsatile' movements of mitochondria between the cortex and the centre of the oocyte. The changes in rates of translation of some subsets of transcripts, and the many post-translational modifications described earlier, would form part of this sequence. However, GVBD alone is not adequate to trigger the unfolding of the oocyte programme, since conditions exist in which the nuclear events of oocyte maturation can be induced to occur without all the attendant cytoplasmic changes (Warnes *et al.* 1977). Such oocytes are not viable (Moor & Trounson, 1977). Thus a combination of interaction of cytoplasmic and nuclear triggers may be required for complete development. Interestingly, in light of the striking dynamic changes in distribution of cellular organelles during oocyte maturation to second metaphase, some of the protein kinases responsible for the post-translational changes are observed in association with a detergent-resistant, presumptive cytoskeletal fraction of the oocyte (Van Blerkom, 1984*a*). These observations have led to the suggestion that there is strict spatial as well as temporal programming over this period of development and that this may underlie the complex movement of organelles (Van Blerkom, 1984*a*).

When the oocyte programme reaches second metaphase, there is a pause during which protein synthesis continues but does not show appreciable qualitative changes. If fertilization or parthenogenetic activation occurs, the egg enters into the *activation programme* described earlier. If fertilization does *not* occur, the egg nonetheless manifests essentially the same changing qualitative pattern of biosynthetic activity. However, although the sequence of changes is very similar to that which occurs normally in fertilized eggs, it takes two to three times longer to complete. Careful examination reveals that these changes are not due to covert parthenogenetic activation of the eggs (Van Blerkom, 1983, 1981*b*; Pratt *et al.* 1983; Howlett & Bolton, 1985). Thus, elements of the oocyte programme proceed regardless of activation, but the occurrence of most is accelerated by fertilization. It is tempting to speculate, and possible to test, that the catalytic effect at activation might reside in the changing $Ca^{++}$ and ionic fluxes at fertilization (Cuthbertson, Whittingham & Cobbold, 1981; Igusa, Miyazak & Yamashita, 1983), in a manner analogous to that described for sea urchin eggs (Winkler, Steinhardt, Grainger & Minning, 1980).

Since elements of the oocyte programme run on regardless of the time of fertilization, it is of interest to ask whether GVBD, and not fertilization, provides the clock that times entry into the second cell cycle and the processes leading to activation of the embryo's own genes. That this is not the case is shown by examination of eggs fertilized at different times after the stimulus to meiotic maturation, which reveals that it is the time of sperm entry, and not the time of meiotic resumption, that provides the starter's gun in the race to division and gene activation (Howlett & Bolton, 1985). In summary then, the first 30 or so hours of mouse development appear to involve two programmes, each activated in sequence, and probably via rather non-specific changes in the cytoplasmic milieu, that cause major cascades of post-translational modification and mRNA

selection from presynthesized maternal stores. We have at present only the slightest insight into the elements of causality within this sequence, but the definition of the precise temporal sequence makes an intelligent approach to that analysis feasible.

*(c) The transition from a maternal to an embryonic programme*

The first identifiable embryonic transcripts appear only in G1 of the second cell cycle (Bolton *et al.* 1984) and evidently code for the heat-shock proteins 68 and 70 (Bensaude *et al.* 1983). Interestingly, the earliest developmentally regulated genes to be activated transcriptionally during induction of *Dictyostelium* differentiation are associated with a 16-base sequence 70 bases upstream that is highly homologous with the sequence reported to be responsible for induction of heat-shock genes in *Drosophila* (Zucker *et al.* 1983; Pelham, 1982). Appearance of heat-shock mRNAs during normal development of *Drosophila* has also been reported (Zimmerman, Petri & Meselson, 1983). In the mouse this initial expression of heat-shock genes is followed by DNA replication, expression of a major set of embryonic genes at the mid 2-cell stage (including actin, histone and IAP mRNAs) and the disappearance of maternal mRNA (Flach *et al.* 1982; Bolton *et al.* 1984). Despite the loss of maternal mRNA, maternally inherited effects on development persist well beyond the 2-cell stage (reviewed by McLaren, 1979; Magnuson, 1983). Presumably these effects are mediated either by a small subset of stable maternal mRNAs or by some other inherited feature that has a longer half life than mRNA. The only example of a maternally inherited cytoplasmic factor that has been indentified is the mitochondrial factor (Mtf) that regulates the expression of the H-2-linked gene *Hmt* coding for the medial transplantation antigen Mta (Fischer Lindahl, Hansmann & Chapman, 1983; Smith *et al.* 1983).

Studies involving the carefully timed application of the RNA polymerase II inhibitor alpha-amanitin suggest that neither the second round of DNA replication nor the breakdown of maternal mRNA are dependent upon embryonic transcription. These events appear to represent terminal features of the post-transcriptional regulatory cascade initiated at fertilization. The synthesis of the heat-shock proteins is not in itself sufficient to allow division to four cells, which also requires the mid-cycle burst of transcription (Flach *et al.* 1982; Bolton *et al.* 1984). The mechanism by which maternal mRNA disappears is unknown. It is probably not necessary to postulate a process of selective destruction in which maternal mRNAs are marked for destruction, because a simple increase in mRNA turnover rates would effectively eliminate maternal mRNA selectively compared with the continuously replenished embryonic transcripts.

The activation of the embryonic genes early in G1 of the 2-cell stage follows closely upon the preceding round of DNA replication. Neither pronuclear fusion nor cleavage are required for activation, as both are inhibited by cytochalasin D which does not interfere with gene activation (Petzoldt, Burki, Illmensee &

Illmensee, 1983; Bolton *et al.* 1984). In contrast if the round of pronuclear DNA replication is inhibited by aphidicolin (S. K. Howlett, in preparation) or if the completion of karyokinesis is inhibited by colcemid after replication is completed (Bolton, 1984) then expression of the embryonic genome is prevented (or severely disturbed) and maternal mRNA is stabilized. These results imply that either the first round of replication, or the first round of karyokinesis, or both, are required for the full transition from maternal to embryonic programmes. This point is discussed further in the next section.

*(d) Control in the embryonic programme(s) for early development*

Control over the timing of gene expression in development (or more accurately over the timing that regulates the availability of a particular developmental option; see earlier) has been the subject of numerous studies (see Satoh, 1982). Time in most, if not all, developmental programmes including the mouse, does not appear to be measured by absolute cell number (Smith & McLaren, 1977), absolute time (Smith & McLaren, 1977) or the number of rounds of cytokinesis (Surani, Barton & Burling, 1981; Kimber & Surani, 1981; Eglitis & Wiley, 1981; Pratt, Chakraborty & Surani, 1981; Petzoldt *et al.* 1983). In many systems some sort of relationship is observed between the expression of the developmental programme and the number of nuclear cycles elapsed (or the related parameter in early development – a change in nucleocytoplasmic ratio). The relationship may appear simple as in *Xenopus*, where a given number of nuclear cycles (twelve) are followed by a given developmental event (the midblastula transition; Newport & Kirschner, 1982), or more complex as in the *Ascidian* embryos in which a given number of nuclear cycles (eight) are required for an event that may only be expressed 2 or 3 cell cycles later (appearance of mRNA for acetylcholinesterase; Meedel & Whittaker, 1979; Satoh & Ikegani, 1981*a*,*b*). The latter example reveals that nuclear changes may be necessary but not in themselves sufficient, and suggests an interaction of a nuclear and a cytoplasmic programme. However, in both cases, regardless of how attenuated the connection between the so called 'quantal' nuclear cycle and the observed outcome of a change in transcriptional activity might be, the presumption is that DNA replication is required for the influence of cytoplasmic agents, whether inherited or positionally signalled, to be exerted. This assumption is not unreasonable, since we know that some changes in chromatin organization that are associated with a change in the potential for gene expression also require (or are greatly facilitated by) DNA replication, *viz* changes in methylation levels and nucleosome restructuring (e.g. Bird, this volume; Leffak, 1984). Replication may also provide the opportunity for access to the enhancer or promoter regulatory sequences inferred from the studies described earlier.

The failure of transcription after inhibition of the first round of DNA replication is at least consistent with the above model. However, it seems unlikely that the mouse embryo conforms to the model proposed for *Xenopus* embryos, in

which, during twelve rounds of division, DNA is replicated in a constant cytoplasmic volume, thus, it is postulated, effectively titrating out inhibitory factors and allowing gene activation. Relative depletion of cytoplasm or addition of DNA led to premature activation (Newport & Kirschner, 1982). In the mouse, there is only one round of replication prior to transcriptional activation, and this round merely restores the ratio that existed in the secondary oocyte only 10–12 h previously. Moreover, although more precise studies need to be undertaken, disturbances of ploidy do not produce the alterations in developmental timing to be expected if titration of a cytoplasmic factor was involved in very early development (reviewed Kaufman, 1983). It seems more likely that the first round of DNA replication may be qualitatively different from ensuing rounds, not an unlikely notion since the female chromosomes have not been replicated for months or years and the male chromosomes for weeks. Moreover, the male chromosomes will have been transcriptionally inactive for several weeks and the female chromosomes for about 36 h. There is also clear evidence that during, or immediately prior to, entry into meiosis the structural organisation of chromatin in the oocyte is modified (McLaren, 1983). In addition, a number of studies indicate that once the first round of DNA replication is accomplished, subsequent developmental events are less immediately dependent upon replication.

Some of these studies are difficult to interpret in detail due to ambiguities of protocol or difficulty in determining the level at which the drugs used were acting (Braude, 1979*b*; Alexandre, 1979, 1982; Cozad & Warner, 1982). However, three studies which used aphidicolin (and in some cases FUdR as well) suggest a measure of independence from nuclear or DNA cycles. Bolton *et al.* (1984) report that inhibition of the second round (2-cell stage) of DNA replication does not affect the subsequent mid 2-cell activation of embryonic genes. Dean & Rossant (1984) delayed the fourth round (8-cell stage) of DNA replication by 8 h, and found that blastocoel formation occurred on time (or slightly earlier), but with half the cell number (25 versus 63) and therefore presumably after one less nuclear replication cycle (although this was not tested directly). Smith & Johnson (1985) inhibited the third (4-cell) and fourth (8-cell) cell cycles by continuous exposure to aphidicolin. Despite complete suppression of DNA replication, cell polarization and flattening occurred on time (or slightly earlier) at the mid to late 8-cell stage. Moreover, at the time when control embryos were dividing from 8- to 16-cells, aphidicolin-treated embryos appeared to show decompaction and cell surface modifications similar to those observed in dividing cells. Thus some sort of developmental clock had run through two complete cell cycles in the absence of DNA replication.

The conclusion to be drawn from these observations, albeit provisional in the light of the rather fragmentary pattern of experiments, must be that either the second and later DNA replication cycles are irrelevant to the timing of early developmental events, or that for any of the developmental events analysed a

'quantal' replication cycle must exist some time previous to those examined, even though its effects are not observed for several cell cycles.

Thus far, we have reviewed only the evidence concerning the relationship between DNA replication and developmental reprogramming. However, implicit in the discussion is the assumption that at replication, chromatin is also reprogrammed. What evidence is there as to the nature of this reprogramming? The very limited amounts of DNA that can be recovered from early mouse embryos makes direct analysis of changes in its properties or structure extremely difficult. However, two lines of indirect evidence are available to suggest that major changes might occur.

Reprogramming of the sperm-derived chromatin during the 1-cell stage could occur during its decondensation and reconstitution with oocyte-derived histones. An imprinting effect of the recent passage of the male chromatin through a prolonged quiescent, condensed phase is implied from various observations. Thus, in female embryos the paternally derived X chromosome is inactivated preferentially in the tissues derived from trophectoderm and primary endoderm (West, Frels, Chapman & Papaioannou, 1977; Takagi, Wake & Sasaki, 1978; Harper, Fosten & Monk, 1982). Moreover, the inactivation state of the X chromosome in both spermatozoa and primary endoderm betrays differences from that of the X chromosome in derivatives of the primary ectoderm (Kratzer *et al.* 1983). In addition, a paternally derived pronucleus appears to be more efficient at rescuing parthenogenetically activated eggs from developmental arrest than a maternally derived pronucleus and may do so via effects on the development of trophectodermal and/or endodermal components of the conceptus (Surani, Barton & Norris, 1984). Further evidence for differences between male- and female-derived pronuclei (and possibly therefore for differences in chromatin/genes, although this was not demonstrated directly) comes from the observation that $T^{hp}$ is only lethal when transmitted through the maternal nucleus and not the paternal nucleus (McGrath & Solter, 1984). Taken together, these observations suggest that at least some reprogramming in at least some tissue lineages must be occurring in early development but the location and timing of these changes remain to be determined.

A different line of evidence leads to a similar conclusion. Thus, experiments involving gene injection or infection by retroviruses (see earlier) suggest the presence of a gene inactivation mechanism that is correlated with a *de novo* methylase activity in the very early embryo. Integrated DNA ceases to be expressed and is found subsequently to have been methylated. The presumption is that this methylation occurred at, or shortly after, integration and was responsible for the inactivation of expression. It is not clear how early in development the *de novo* methylation occurs and indeed there is evidence to suggest that methylation changes form a relatively late part of gene regulation (see experiments by Groudine & Weintraub earlier). However, attempts to

measure methylation levels of total DNA, or of major and minor satellite repetitive sequences within the DNA, suggest that 2- to 8-cell cleaving embryos, morulae, inner cell mass derivatives and their equivalent, the embryonal carcinoma cell lines, are all heavily methylated (Singer, Roberts-Ems, Luthardt & Riggs, 1979; Manes & Menzel, 1981; Chapman *et al.* 1984). Since the same repetitive sequences both in spermatozoa and oocytes are reported to be relatively undermethylated (Chapman *et al.* 1984; Ponzetto-Zimmerman & Wolgemuth, 1984; Sanford *et al.* 1984), the presence of a *de novo* methylase activity very early in cleavage might be inferred. However, single copy sequences may behave differently from repetitive sequences. Several reports suggest a *high* level of methylation in single-copy sequences and interspersed repetitive DNA in spermatozoa (Ehrlich *et al.* 1982; Ponzetto-Zimmerman & Wolgemuth, 1984; Sandford *et al.* 1984), although dispersed repetitive sequences in oocytes were reported as being undermethylated (Sanford *et al.* 1984). Thus, we do not know the earliest developmental stage at which *de novo* methylation can operate, what the precise substrate for methylation might be, and in particular whether the 1-cell stage is unique. Nor do we know whether replication of DNA is required for *de novo* methylation. The delamination of trophectoderm, and subsequently of endoderm, from the ICM is marked by relative hypomethylation in these tissues and their derivatives (Manes & Menzel, 1981; Chapman *et al.* 1984). This cell lineage related undermethylation applies to both repetitive and single copy genes (J. Rossant, J. Sanford & U. H. Chapman, personal communication). The significance of this observation is uncertain. It is, however, important to re-emphasize that methylation levels are unlikely to be the primary or sole determinant of the state of chromatin, but rather in general show an inverse correlation with expression (or capacity for expression).

It is clear that despite the inactivation of introduced exogenous genes, and their associated *de novo* methylation, gene activity and the production of new transcripts is occurring in the early embryo. Presumably therefore a class of early acting embryonic genes escapes the inactivation process. This could be achieved by the presence of oocyte factors that protect identifier gene sequences, by the presence of strong enhancer sequences that resist the inactivation process or react paradoxically to it (Tanaka, Apella & Jay, 1983), or by a combination of both. Hopefully the development of probes for genes expressed in early development (such as heat-shock genes and IAP genes), and their application to the programmed changes now being defined with some precision, will make it possible to understand how chromatin behaves when developmental decisions are being made.

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