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Recent advances in our understanding of the temporal control of early embryonic development in amphibians

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SUMMARY

Recent studies on temporal control of early amphibian development are reviewed. It is becoming clear that the development of an embryo is not timed by a single clock set in motion at fertilization, instead each developmental event seems to be timed by its own clock-like mechanism. The timing of developmental events is rigidly determined within embryonic cells, and usually can not be altered experimentally. One exception, however, is the timing of midblastula transition in amphibian embryos; recent studies have shown that its timing is regulated by the nucleocytoplasmic ratio. Several developmental events, particularly those associated with transcriptional activities, require DNA replication prior to their occurrence, suggesting an intimate relationship between DNA replication cycles and their onset. On the other hand, there are many other developmental events where timing is not controlled by the number of cell divisions, DNA replication cycles, or the nucleocytoplasmic ratio. Cytoplasmic machinery with autonomous oscillatory properties is thought to be involved in the timing of these events.

INTRODUCTION

Time is an indispensable element in any interpretation of embryological development. It is obvious that a better understanding of the temporal control of development will bring about a more profound interpretation of these processes including the regulation of gene activities in differentiating cells and control of morphogenetic movements in embryonic cells constructing spatial patterns. Recently, attention has been drawn to the question, 'How is it that cells in a developing embryo do the right thing *at the right time*?' or 'How does a developing embryo measure the time flow after fertilization and what determines the timing of developmental events, each of which starts very much precisely within embryonic cells?' (for reviews see Snow & Tam, 1980; Satoh, 1980, 1982, 1984). Since the turn of this century, much data at the level of not only experimental morphology but also molecular biology, has accumulated on the early embryonic development of amphibians. The large size of amphibian eggs makes them advantageous for certain manipulations such as bisection of eggs into nucleated and non-nucleated fragments, extraction of egg cytoplasm to alter the nucleocytoplasmic ratio, and injections of exogenous

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materials into eggs. During the last decade, many studies have been carried out in order to elucidate the temporal control of early amphibian development. Our knowledge of the timing mechanisms that form the basis for temporal control, which will be reviewed and discussed in this paper, has been greatly increased.

TIMING MECHANISMS RESPONSIBLE FOR EARLY DEVELOPMENTAL EVENTS

(*a*) *Midblastula transition.*

Amphibian eggs undergo 11 (axolotl) or 12 (*Xenopus, Cynops*) rapid synchronous cleavages followed by a transition to slower asynchronous cleavages. Accompanying this change or midblastula transition (Signoret & Lefresne, 1971; Gerhart, 1980), distinct G_1 and G_2 phases of the cell cycle appear, and the blastomeres become motile and active in RNA synthesis. There are a number of experiments which indicate that the ratio of nuclear to cytoplasmic volume may be closely associated with the initiation of the midblastula transition (e.g., Chulitskaia, 1970; Signoret & Lefresne, 1973; Landström, Lø vtrup-Rein & Lø vtrup, 1975). The causal relationship between the nucleocytoplasmic ratio and the timing of midblastula transition has been clearly revealed by recent studies of Kobayakawa & Kubota (1981) and Newport & Kirschner (1982*a,b*).

Kobayakawa & Kubota (1981) obtained newt egg fragments with a half or a quarter of their normal cytoplasmic volume by cutting a fertilized, unsegmented egg into two or four equal-sized parts. Asynchronous cleavage started one or two divisions earlier in half or quarter embryos compared with whole embryos. Newport & Kirschner (1982*a*) have shown that there is a causal relationship between the onset of the midblastula transition in *Xenopus* embryos and the nucleocytoplasmic ratio but not with the number of cell divisions, the number of rounds of DNA replication, or the elapsed time since fertilization, by doing the following experiments. (i) If cleavages are inhibited by treatment with cytochalasin B or by centrifugation, RNA transcription is activated at the precise time that new synthesis is seen in dividing embryos used as controls. (ii) By partial constriction of a fertilized egg, they obtained half embryos with different cleavage schedules. However, both advanced and retarded sides underwent midblastula transition after the same number (11 but not 12) of cleavages, although the transition in the advanced side took place two cell cycles earlier than in the retarded side which received one of the nuclei of the advanced half after 2 cleavage cycles. (iii) In artificially induced polyspermic eggs which received seven to ten sperm nuclei, the midblastula transition could be induced prematurely two divisions before the transition occurred in diploid embryos. Moreover, they (Newport & Kirschner, 1982*b*) have shown that when a plasmid containing a cloned gene coding for yeast leucine tRNA was injected into cleaving eggs, it became transcriptionally active at the midblastula transition. The suppression of transcription prior to the midblastula transition could be reversed by addition of competing DNA. The amount of DNA needed to induce

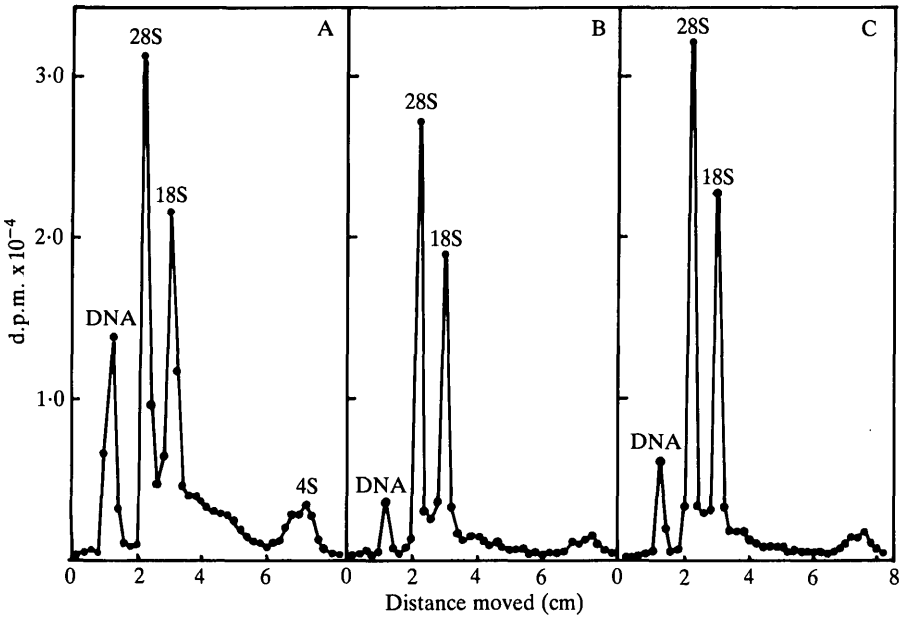

Fig. 1. Gel electrophoretic profiles showing the synthesis of rRNA in cleavage-arrested *Xenopus* embryos. Embryos were treated with cleavage inhibitors at the 16-cell stage. After 5 h, when the control embryos reached the late blastula stage and the rRNA synthesis was expected to begin, labelling with 40 μCi ml^{-1} of [^{3}H]guanosine (5 Ci mmol^{-1}) was started. It was continued for 7 h until the control embryos reached stage 11. Then, RNA was extracted from fifteen embryos and analysed. One third of the extracted RNA was loaded on each gel. (A) control embryos; (B) colchicine-arrested embryos; (C) cytochalasin-treated embryos. The peaks of radioactivity are identified, in order of increasing mobility, as DNA (including 40S pre-RNA), 28S rRNA, 18S rRNA, and 4S RNA on the basis of mobility, gel-staining pattern and sensitivity to alkali treatment. The synthesis of 4S RNA and heterogeneous RNA is more or less suppressed in the cleavage-arrested embryos. The amount of labelled DNA in the arrested embryos,especially in the colchicine-treated embryos, is much less than that of the control. However, in all examples, active synthesis of 28S and 18S rRNA is occurring. (From Takeichi, Satoh, Tashiro & Shiokawa, 1985).

premature transcription was equal to the amount of nuclear DNA present after 12 cleavages. They have proposed that the midblastula transition is triggered by the DNA through the titration of suppressor components present in the eggs (Newport & Kirschner, 1982*b*); this mechanism will be discussed later.

(*b*) *Gastrulation.*

It has been reported that neither the number of cell divisions, the number of DNA replication cycles, nor the nucleocytoplasmic ratio are critical factors for the timing of initiation of gastrulation in amphibian embryos (e.g. Holtfreter, 1943; Smith & Ecker, 1970; Cooke, 1973; Signoret & Lefresne, 1973; Kobayakawa & Kubota, 1981). Recently Boterenbrood, Narraway & Hara (1983) have observed the cleavage pattern of *Xenopus* embryos by means of time-lapse cinematography.

The beginning of cell cycle lengthening varies quite a lot between individual embryos, it may start in either cycle 10, 11 or 12. However, the first lengthened cycle shows no correlation with the corresponding pregastrulation period in either duration or lengthening. Instead, the duration of cycle 13 is inversely correlated with the period elapsing from the beginning of this cycle to the onset of gastrulation which occurs in cycle 14 or 15; that is, as the lengthening of cycle 13 is greater, gastrulation starts relatively earlier.

(*c*) *rRNA synthesis.*

The synthesis of rRNA in *Xenopus* embryos begins during, or a short time after the midblastula transition. However, the timing of rRNA synthesis or rRNA gene expression is not dependent on the nucleocytoplasmic ratio, the number of constituent cells, cleavage cycles, or DNA replication cycles. This has been shown in embryos manipulated in four different ways: cytochalasin-arrested embryos, colchicine-blocked embryos, podophyllotoxin-inhibited embryos, and embryos with reduced cytoplasm (Takeichi, Satoh, Tashiro & Shiokawa, 1985; Shiokawa *et al.* 1985). If *Xenopus* embryos at early cleavage stages are treated with cytochalasin B or colchicine, cleavage was completely blocked. The amount of rDNA in colchicine-blocked embryos, determined by dot-hybridization was less than 1/25 of that in normal control embryos. However, as shown in Fig. 1, rRNA synthesis in these cleavage-arrested embryos began at almost the same time it did in normal embryos. In addition, the nucleolar formation in podophyllotoxin-arrested embryos began at the normal time. Furthermore, the onset and rate of rRNA synthesis in embryos from eggs in which about 40–50% of cytoplasm had been extracted, was not appreciably different from that of control embryos.

(*d*) *Fibronectin synthesis.*

Recently temporal regulation of fibronectin synthesis during early *Xenopus* development has been studied by Lee, Hynes & Kirschner (1984). Fibronectin levels and fibronectin synthesis rates increase following the midblastula transition. Since α-amanitin did not block this increase, fibronectin synthesis is dependent on activation of maternal RNA for fibronectin. Eggs activated by pricking start their developmental program but do not undergo the midblastula transition since the nucleocytoplasmic ratio never reaches that of a 4000-cell embryo. In such activated eggs, however, fibronectin synthesis begins at almost the same time it does in control embryos. Therefore, the timing mechanism responsible for the increase in fibronectin is not dependent on fertilization, cell division, or the nucleocytoplasmic ratio.

(*e*) *DNA ligase in axolotl eggs.*

Unfertilized axolotl eggs contain a light form (6S) of DNA ligase. When fertilized eggs enter cleavage, however, this form of the enzyme is replaced by a heavy

form (8·2 S), characteristic of developing embryos (David, Vinson, Lefresne & Signoret, 1979; Carre, Signoret, Lefresne & David, 1981). Since this replacement is sensitive to cycloheximide, actinomycin D, α-amanitin, and injury of the female pronucleus by u.v. irradiation, this change involves *de novo* protein synthesis and transcription of an intact maternal but not the paternal genome (Signoret, Lefresne, Vinson & David, 1981). By doing gamma-ray irradiations at different times after activation, it has been established that the female nucleus has to be intact up to 3·5 h after activation for the replacement of the ligase to occur. Inhibition of the first DNA replication by treatment with aphidicolin or arabinosyl cytosine completely blocked this change (Lefresne, David & Signoret, 1983). The inhibitory effect of arabinosyl cytosine can be reversed by treatment with deoxycytidine, a competitive inhibitor of arabinosyl cytosine. These results clearly indicate that DNA replication and/or new chromatin synthesis is a prerequisite for the expression of new genomic activity.

THE CYTOPLASMIC CLOCKS AND THE REGULATION OF THE CELL CYCLE

(*a*) *Cytoplasmic clocks.*

It has been shown that non-nucleated cytoplasmic fragments of certain invertebrate eggs show cyclic changes in their shape or in their physiological properties with a temporal pattern very similar to that of the changes accompanying the division cycle of normally fertilized eggs (references see Satoh, 1982). Recent work has established that autonomous cyclic activity occurs in the cytoplasm of amphibian eggs as well. Preceding each of the first few cleavages in amphibians, the eggs undergo a contraction of their cortex called the 'surface contraction wave', which can be visualized by a time-lapse cinematography (Hara, 1971; Yoneda, Kobayakawa, Kubota & Sakai, 1982). Amphibian eggs deprived of the vitelline membrane show a periodic rounding up and relaxation at each cleavage cycle. These phenomena can be used as markers for monitoring cyclic activity in non-nucleated egg fragments. Sawai (1979) has observed a set of periodic changes in the tension and height of the cortex in non-nucleated fragments of the Japanese newt *Cynops pyrrhogaster*. Hara, Tydeman & Kirschner (1980) and Sakai & Kubota (1981) have reported that a non-nucleated *Xenopus* egg fragment, produced by constricting a fertilized egg, shows periodic contraction waves and a rounding-up and relaxation, with approximately the same frequency as those seen in cleaving (nucleated) fragment. All of these observations clearly indicate that there is an autonomous oscillatory regulator of the cell division cycle, residing in the cytoplasm of the egg. Neither the cycle of DNA replication nor the cycle of centriole duplication, mitosis, and cytokinesis is required for the expression of these periodic cortical activities (Kirschner, Gerhart, Hara & Ubbels, 1980).

An important demonstration of Sakai & Kubota (1981) is that the rounding-up intervals of the non-nucleated fragments of *Xenopus* eggs were slightly but

definitely longer than the cleavage intervals of the nucleated fragments and whole eggs. The difference in the intervals of cyclic changes between nucleated and non-nucleated fragments was carefully examined by Shinagawa (1983). He showed that non-nucleated fragments had a cycle of longer duration than the normal cleavage cycle (about 30 %) irrespective of the procedure used to obtain non-nucleated egg fragments. Injection of colchicine or vinblastine into *Xenopus* eggs induced a lengthening in the cycle of normal eggs as well as in nucleated fragments similar to that observed in non-nucleated fragments (Shinagawa, 1983). In addition, transplantation of nuclei derived from gastrula cells could accelerate the cytoplasmic cycle of *Xenopus* non-nucleated egg fragments (Sakai, 1982). Furthermore, when sperm treated with Triton X100 were injected and incubated during the rounding-up phase of the non-nucleated *Xenopus* egg fragment, swollen vesicular nuclei were very frequently observed, whereas no such structures were found when they were incubated during the relaxing phase (Sakai & Shinagawa, 1983). Sakai & Shinagawa (1983) have postulated that the egg cytoplasm plays a more important role than the mitotic apparatus including the nucleus in regulating the phase and length of the cell cycle, as classically pointed out by Moore (1933). They have also suggested that the periodicity intrinsic to egg cytoplasm is modulated by the assembly and disassembly of the mitotic apparatus.

The cell cycle length during synchronous cleavages is rigidly determined for each amphibian species. However, it has been shown that the duration of the cleavage cycle can be altered by reciprocal cytoplasmic transfers between a species with a fast (*Xenopus*) and a slow (*Pleurodeles*) cell cycle length (Aimar, Delarue & Vilain, 1981). Injection of *Xenopus* egg cytoplasm, for example, induces precocious cleavage furrows in activated *Pleurodeles* eggs and leads to nearly a 30 % shortening of the cell cycle compared with control eggs. This activity or the activity of 'cleavage timing system (CTS)' was found only in the egg cytoplasm but not in the karyoplasm. It was first detected in the cytoplasm of maturing oocytes at the stage of germinal vesicle breakdown but the cytoplasmic property was not dependent on MPF (Aimar *et al.* 1981). More recently these investigators have shown that the cytoplasmic components responsible for CTS activity are found in the clear supernatant obtained by centrifugation of *Xenopus* eggs at 120000 *g* for 1 h (Aimar, Vilain & Delarue, 1983). In addition, CTS activity is retained in two distinct fractions when the supernatant is fractionated by gel filtration. Preliminary characterization of these fractions indicated that the factors are thermostable and resistant to RNase treatment but the CTS activity is abolished after protease treatment. These results suggest that there are cytoplasmic components with a protein-like nature which determine the endogeneous cleavage-cycle length; they also suggest that the components from different species share several common properties whereby they can advance or retard the cleavage cycles of other species.

Shinagawa (1985) has recently done a set of experiments that deal with the localization of the factors responsible for the periodic activities associated with the cleavage cycle. When fertilized *Xenopus* eggs are bisected along the boundary line

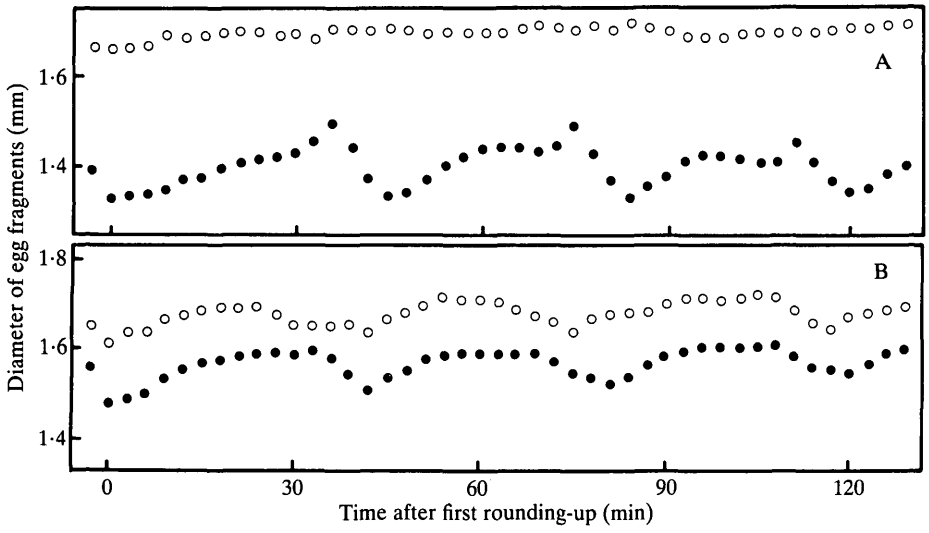

Fig. 2. Changes in the diameter of animal halves (●), prevented from undergoing mitosis with colchicine, and the vegetal halves (O) formed by bisecting the fertilized but unsegmented *Xenopus* eggs after rotation through 90°. In (A) bisection was carried out immediately after rotation, whereas in (B) bisection was done 30 min after rotation, allowing the relocation of cytoplasm by gravity. In (A) a typical, periodic rounding-up activity can be seen only in the animal half, but in (B) the activity is detected not only in the animal half but also in the vegetal half (From Shinagawa, 1985).

between the animal and vegetal halves immediately after being rotated through 90°, the resulting animal halves, though prevented from cell division by colchicine treatment, show periodic rounding-up and relaxation as previously observed for enucleated egg fragments, but the vegetal halves do not (Fig. 2A). However, if eggs are separated into animal and vegetal halves following incubation for 30 min after a 90° rotation, thereby allowing the relocation of the endoplasmic components in the rotated eggs under the force of gravity, not only the animal halves but also the vegetal halves show typical cyclic rounding-up (Fig. 2B). These results suggest that the cytoplasmic factors inducing the periodic activities are not distributed uniformly throughout the egg but localized mostly in the animal hemisphere and that these factors can be moved by gravity from the animal to the vegetal hemisphere of *Xenopus* eggs. These experimental results have been confirmed in the urodele *Cynops* (Shinagawa, unpublished data). When fertilized newt eggs are bisected into animal and vegetal halves soon after being rotated through 90°, the duration of cleavage cycles of the vegetal halves (presumably due to a sperm nucleus incorporated by physiological polyspermy) is about 1·5 times longer than that of animal halves. However, if the eggs are retained for 30 min after a 90° rotation and then cut into animal and vegetal halves, the vegetal halves had the same cleavage rhythm as the animal halves. If the cytoplasmic components, whose localization has been studied by Shinagawa (1985) are the same as those associated with CTS activity investigated by Aimar *et al.* (1981, 1983), the cytoplasmic clock responsible for

determining cleavage cycle length may be localized in the animal endoplasm which can be displaced by gravity.

(*b*) *The regulation of cell cycle.*

Details of recent studies on the regulation of cell cycles in amphibian eggs and early embryos is discussed in the chapter by Ford in this volume. Here I will briefly mention the significance of MPF (maturation promoting factor) in the regulation of cell cycle.

MPF, which was first detected in unfertilized amphibian eggs, is thought to be the cytoplasmic agent responsible for the initiation of the meiotic or mitotic phase of the eukaryotic cell cycle (for reviews see Masui & Clarke, 1979; Maller & Krebs, 1980). MPF is found not only in amphibian oocytes (eggs) and starfish oocytes (eggs) but also in cultured mammalian cells and *cdc* mutants of yeast (Kishimoto & Kanatani, 1976; Sunkara, Wright & Rao, 1979; Weintraub *et al.* 1982). Its activity is not species specific; MPF from different phyla are found to be equally effective (Kishimoto, Kuriyama, Kondo & Kanatani, 1982). The ubiquitous presence of MPF in mitotic and meiotic cells suggests that MPF has an important role in regulation of cell cycle. MPF of *Xenopus* eggs has been partially purified; it behaves as a protein with a molecular weight of 100 KD (Wasserman & Masui, 1976; Drury, 1978; Wu & Gerhart, 1980). In addition, it has been shown that partially purified MPF can initiate the early events of metaphase when injected into cycloheximide-inhibited *Xenopus* oocytes (Miake-Lye, Newport & Kirschner, 1983). Recently, Newport & Kirschner (1984) have successfully demonstrated: (i) without protein synthesis, addition and removal of MPF can drive the mitotic cycle in *Xenopus* eggs, including nuclear membrane breakdown and reformation, chromosome condensation and decondensation, and suppression and initiation of DNA replication on endogenous DNA and on injected plasmid templates; (ii) M-phase arrest induced by CSF (cytostatic factor) blocks an endogenous cytoplasmic cell-cycle oscillator and causes the stabilization of MPF activity; (iii) the oscillator can be restarted by injection of Ca^{2+}; and (iv) the effects of MPF are on the chromatin template and not on the replication machinery. Based on the results, they have postulated that in *Xenopus* embryos cell-cycle events of the nucleus including DNA replication and mitosis, are controlled by the level of MPF activity, which is driven by or may be part of an autonomous cell-cycle oscillator (Newport & Kirschner, 1984).

In addition to such a significant role of MPF in the regulation of cell cycle, the level of MPF activity changes cyclically coincidentally with division cycles. This was first shown by Wasserman & Smith (1978) in cleaving *Xenopus* eggs; a high activity of MPF appears at metaphase, exhibiting cyclic behaviour that is synchronous with the cell cycle of blastomeres. The cell cycle dynamics of MPF in *Xenopus* oocytes and eggs has recently been studied by Gerhart, Wu & Kirschner (1984); they reported that MPF is amplified by post-translational mechanisms operating on a pool of MPF precursor at the first maturation division phase, but in the absence of

protein synthesis MPF never reappears at the second meiotic division and the following mitotic cycles. They also reported that the repeated reciprocal cycling of MPF and its inactivating agent does not require the effective completion of spindle formation, mitosis, or cytokinesis. Furthermore, with respect to the oscillatory activity of MPF, an intriguing experimental result has been reported by Masui (1982). If clear extracts are prepared from dejellied *Rana pipiens* eggs by centrifugation at 150 000 *g* for 2 h, and stored at 0 °C for 2–3 weeks, MPF activity first disappears from the extracts on day 3 or 4, but reappears again on the following day and persists for 2 or 3 days at high levels before disappearing again. The cycle of MPF activity in extracts is repeated fairly regularly during storage of the extracts. In his recent review of cyclic-cell coordinating events, Masui (1985) has pointed out that the MPF cycle may be an autonomous process and that it appears to regulate other cyclic changes; he has proposed theoretical models to explain how the MPF oscillator works in the cell.

DISCUSSION AND PERSPECTIVE

One of the reasons it is difficult to study timing mechanisms is that a decision regarding the initiation of an event usually takes place, inside embryonic cells, several hours (or days) before the event actually occurs. In addition, the timing of developmental events are rigidly determined within embryonic cells, and usually they are difficult to alter experimentally. In spite of these problems, recent studies have provided a considerable amount of information concerning the temporal control of early embryonic development. First, it is now obvious that not every developmental event is timed by a single clock set in motion at fertilization, as was predicted previously (Snow & Tam, 1980; Satoh, 1980, 1982); the timing of the midblastula transition, for example, is determined by the nucleocytoplasmic ratio, whereas the initiation of rRNA synthesis or of fibronectin synthesis is not timed by the nucleocytoplasmic ratio. This suggests that we should first study the timing mechanism of each developmental event independently and then organize them into some kind of a system in order to provide an overall understanding of the temporal control of embryonic development.

Second, it has been clearly shown that the onset of the midblastula transition in amphibian embryos depends on reaching a critical ratio of nucleus to cytoplasm (Kobayakawa & Kubota, 1981; Newport & Kirschner, 1982*a*,*b*). This may also explain the onset of asynchronous cleavages in other animals (e.g. Mita, 1983; Mita & Obata, 1984). When the timing of developmental events is based on a critical nucleocytoplasmic ratio, this necessarily means that DNA replication and cytokinesis must play a significant role in making this mechanism work. Newport & Kirschner (1982*b*) have proposed a titration model in order to explain the activation of transcription governed by RNA polymerase II which occurs at the midblastula transition. According to the model, the unfertilized egg contains a large cytoplasmic pool of a factor which is bound to chromatin and is capable of suppressing tRNA transcription. At the end of each synchronous round of DNA synthesis

the total amount of DNA in the whole egg is doubled and the new chromatin titrates a portion of the factor from the cytoplasmic pool. The depletion process continues until the completion of round 12 of DNA synthesis, at which time the cytoplasmic pool is depleted. After cleavage 12 the factor bound to DNA becomes diluted with each subsequent round of DNA synthesis, and this dilution process allows for the activation of developmentally programmed transcription (Newport & Kirschner, 1982*b*). Therefore, the exploration of the molecular nature of the suppressor and the manner how the suppressor interacts with the chromatin may be the subject of further studies.

Third, as in the case of DNA ligase in axolotl eggs, it has been shown that several developmental events, particularly those associated with transcriptional activities of the genome, require DNA replication in order to occur, suggesting a close relationship between rounds of DNA replication and the timing of developmental events. This category may include the development of acetylcholinesterase in muscle cells of ascidian embryos (Satoh & Ikegami, 1981*a*,*b*; Mita-Miyazawa, Ikegami & Satoh, 1985), differentiation without cleavage in annelid eggs (Alexandre, De Petrocellis & Brachet, 1982) and probably the synthesis of stage-specific polypeptides in early mouse embryos (Petzoldt *et al.* 1981; Bolton, Oades & Johnson, 1984; Petzoldt, 1985). In order to explain why DNA replication is necessary for the occurrence of some developmental events, at least two possible molecular mechanisms should be addressed. The first is a possibility that some stepwise changes take place, during each round of DNA replication, in the DNA itself. DNA could count its replication number via some of modification such as DNA methylation at specific sites (Holliday & Pugh, 1975; Razin & Riggs, 1980; Satoh, 1984). Another possibility is that during a certain number of DNA replications gradual modifications might occur in the chromatin conformation. Irrespective of whether or not cytoplasmic components (or determinants) exist that are needed for specific gene expression, during a quantal DNA replication cycle, a specific, programmed changes may occur in DNA or in chromatin that bring about specific gene expression.

It has long been thought that genomic activities are involved in temporal control of embryological development. Very recently several 'heterochronic' mutants which affect only the temporal sequence of cell fates but not the formation of spatial pattern have been isolated in the nematode *Caenorhabditis elegans* (Ambros & Horvitz, 1984). The alternations caused by mutations in the gene *lin-29*, for example, act at restricted times on specific cell types; they are limited to certain cuticle-forming cells of the fourth larval stage. The way in which these heterochronic genes act to regulate the temporal sequence of developmental events during the normal embryogenesis has not yet been studied. However, heterochronic mutants may be an attractive experimental system for studying the temporal control of development.

Finally, it is now obvious that there are many other developmental events where timing is not controlled by the number of cell divisions, rounds of DNA replication,

or the nucleocytoplasmic ratio. In addition to gastrulation, rRNA synthesis, and fibronectin synthesis in amphibian embryos, the formation of micromeres in sea urchin embryos (Rustad, 1960; Dan & Ikeda, 1971), gastrulation in starfish embryos (Mita, 1983), and blastocyst formation in mouse embryos (e.g. Smith & McLaren, 1977; Pratt, Chakraborty & Surani, 1981; Dean & Rossant, 1984) may be categorized as such events. It has been proposed that cytoplasmic clocks may be involved in the timing mechanisms of such developmental events (e.g. Painter, 1915; Rustad, 1960; Dan & Ikeda, 1971; Satoh, 1982). The term 'cytoplasmic clock' was first defined by Hara *et al.* (1980) as a biological clock which exists in the cytoplasm or cortex of animal eggs and operates independently of the nucleus or centrioles and which may be involved in *regulating the timing of the cell cycle.* A cytoplasmic clock in this meaning may be identical to the 'cleavage timing system' of Aimar and his colleagues (Aimar *et al.* 1981) and also may be one of the 'independent timers' proposed by Mitchison (e.g., Mitchison, 1984). In addition, the term 'cytoplasmic clock' could be used in wider sense as a developmental clock which may be involved in *counting the time flow and/or determining the timing of initiation of some developmental events* (Satoh, 1982, 1984).

As has been shown in this paper, the existence of cytoplasmic clocks has been completely established. In addition, our understanding of the subcellular mechanisms involved in the regulation of cell cycles in early amphibian embryos has greatly advanced in the last decade, as briefly described in this paper. It is almost certain that MPF is one of the main promoters which drives the cell cycle. The regulation of the cell cycle may be fairly well understood in near future. Moreover, as shown in the studies of Aimar *et al.* (1981, 1983) and Shinagawa (1985), amphibian eggs contain cytoplasmic factors other than MPF, which regulate the length of the cell cycle. With respect to cytoplasmic components, it has recently been demonstrated that substances in the germinal vesicle are required for the establishment of cyclic activities monitored by the change in the cortical stiffness of non-nucleated fragments of starfish oocytes (Yamamoto & Yoneda, 1983; Yamamoto, 1985). Therefore, the following questions might form the basis for future study; (i) What are the molecular properties of the cytoplasmic factors and how do they determine the length of the cell cycle by interacting with MPF? and (ii) How do these cytoplasmic factors interact within embryonic cells to determine the timing of developmental events?

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