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Chromosome replication in early development of *Xenopus laevis*

R. A. LASKEY

CRC Molecular Embryology Group, Department of Zoology, Cambridge CB2 3EJ, U.K.

SUMMARY

Eggs of *Xenopus laevis* contain exceptionally large amounts of materials involved in chromosome replication. This maternal stockpile allows an embryo to produce about 80 000 cells in less than 24 h. The adaptations which achieve this involve the mechanisms of both DNA replication and chromatin assembly.

INTRODUCTION

This paper reviews the specializations which allow embryonic cells of *Xenopus laevis* to replicate their chromosomes faster than *E.coli* can replicate its much smaller chromosome, and about thirty times faster than an adult *Xenopus* cell replicates. How are these rates achieved and sustained in such a way that a *Xenopus* embryo has reached about 80 000 cells by the time a mouse embryo undergoes its first cleavage division? The answers lie in an extraordinary range of adaptations which exaggerate and uncouple normal cellular processes resulting in the slow accumulation and rapid mobilization of a maternal stockpile of materials involved in replication of the chromosome.

THE CELL CYCLE IN EARLY EMBRYOS

Table 1 shows the rate of increase of cell number after fertilization of the *Xenopus* embryo. The first cell cycle lasts for only 1·5 h and the subsequent 11 cycles occupy only 35 mins each (Newport & Kirschner, 1982). Although the progression of the cleavage furrows away from the animal pole creates an impression of asynchrony, these cleavage cycles are synchronous when viewed by time-lapse photography (Hara, Tydeman & Kirschner, 1980). Unlike the cell cycle of adult cells, the cycle in early *Xenopus* embryos lacks detectable G_1 and G_2 phases but consists only of alternating mitosis and S phase (Graham & Morgan, 1966).

The timing of cell cycles in the early *Xenopus* embryo is specified by a cytoplasmic clock which relentlessly triggers cleavage regardless of the state of the nucleus

Key words: cell cycle, DNA replication, G_1 phase, G_2 phase, S phase, mitosis, maturation promoting factor, origins of replication, *Xenopus laevis*

Table 1. *Increase in cell number following fertilization of* Xenopus *eggs*

Hours	Cell number
1	1
2	2
3	8
5	500
10	20 000
20	80 000

(Hara *et al.* 1980). The clock can be monitored by observing the height of the egg because a cortical contraction causes a shape change at the time of each mitosis. The timing mechanism of the clock does not require RNA synthesis or any other function of the nucleus. The periodic contractions continue to occur after the nucleus has been removed surgically. In addition they persist in both halves of a fertilized egg after the egg has been separated into two fragments by a hair ligature. Both the half which receives the zygote nucleus and the enucleate half continue to contract in phase with the clock (Fig. 1 and Hara *et al.* 1980). The contractions are clearly not a secondary consequence of cell division since the enucleate fragment fails to complete division and since they are not disrupted by colchicine or cytochalasin.

Although the mechanism of the clock is not known, Newport & Kirschner (1984) have shown that it can be driven by maturation promoting factor (MPF). MPF has not been identified at the polypeptide level but it appears to be a phosphoprotein with a native molecular weight of about 100 000 daltons (Wu & Gerhart, 1980). It

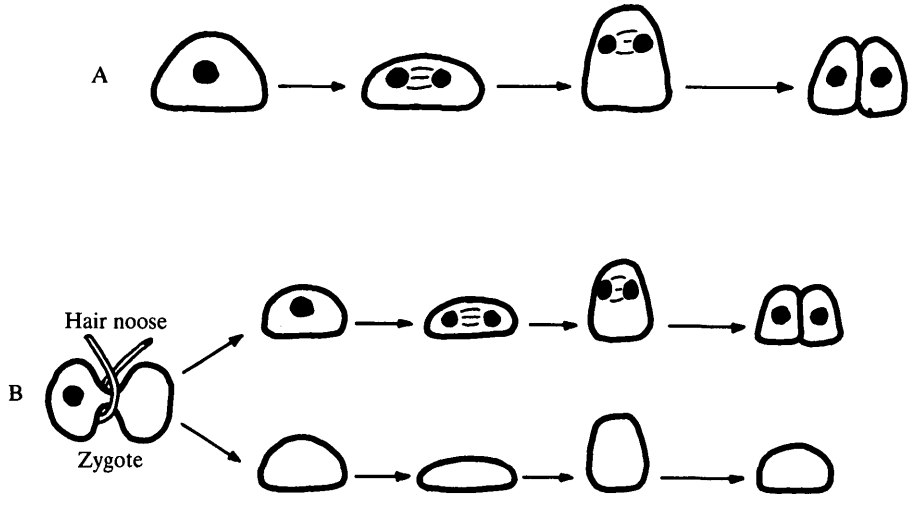

Fig. 1. (A) Cyclical contractions which occur at the times of cleavage of *Xenopus* embryos. (B) The contractions persist in both halves when an egg is divided into nucleate and enucleate fragments by a hair noose. (From the work of Hara, Tydeman & Kirschner, 1980).

was discovered in unfertilized amphibian eggs by its ability to induce oocytes to continue through meiosis from their arrested prophase (Smith & Ecker, 1969; Masui & Markert, 1971). It undergoes rapid autoamplification on injection into oocytes. Newport & Kirschner (1984) used cycloheximide to arrest the cytoplasmic clock at the end of S phase. They then injected partially purified MPF preparations and showed that these caused the arrested eggs to progress into mitosis. Furthermore, as MPF activity decayed, so the injected eggs re-entered S phase, suggesting that cyclical appearance and decay of MPF activity may mediate the cell cycle clock. The mechanism which causes the cyclical appearance and breakdown of MPF remains unknown.

INITIATION OF DNA REPLICATION

Omission of G_1 and G_2 phases contributes to a shorter cell cycle time in early amphibian embryos, but it does not explain how the S phase is also 30 times shorter during cleavage than in adult cells. Callan (1972) has shown that amphibian embryos accelerate their rate of DNA replication by increasing the number of replication forks rather than by increasing the rate of fork progression along DNA (Fig. 2). A similar situation has been shown by Blumenthal, Kriegstein & Hogness (1974) in *Drosophila* embryos, which have total cell cycle times of less than 11 mins and S phases of only 4 mins.

It is clear that, in both *Xenopus* and *Drosophila*, replication is accelerated by increasing the number of initiations on a given length of DNA (Fig. 2), but it is not clear how this is achieved. Several possibilities can be envisaged. First the pattern of initiations could be changed by changing the sequence specificity of initiating factors which would recognize initiation sites. Second, the same factor specificity could be retained, but some potential initiation sites could be made inaccessible, for example by selective chromatin folding. Third it is possible that sequence specificity could be relaxed completely in early embryos so that initiation is random or specified by some structural repeat within the chromosome structure.

Of these possibilities a truly random pattern of initiation appears unlikely. The reason for this is seen most clearly in *Drosophila*. Here the average spacing between initiations is 7·9 kb, the fork movement rate is 2·6 kb min^{-1} and the duration of the

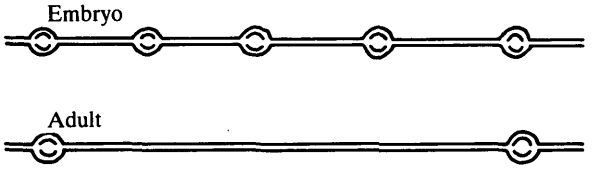

Fig. 2. Schematic representation of the close spacing between adjacent initiations of replication in embryos of *Drosophila* or Amphibia. (From the work of Blumenthal, Kriegstein & Hogness, 1974, and Callan, 1972).

S phase is 4 min (Blumenthal *et al.* 1974). Therefore each bidirectional initiation can synthesize only 21 kb in an entire S phase. If we consider a Poisson distribution around a mean of 7·9 kb then 11 % of all intervals between initiations will fail to complete replication before mitosis. This would result in chromosome breakage and it appears to rule out a truly random distribution, at least in the case of *Drosophila*.

The possibility that the pattern of replication initiation in early embryos is specified by a structural repeat within the chromosome is untested. However it is consistent with two observations. First in *Drosophila* embryos Blumenthal *et al.* (1974) reported that there was a modal distribution of distances between initiations with a tendency for initiations to occur at intervals which were multiples of 3·4 kb. Second, Buongiorno-Nardelli *et al.* (1982) showed that the length of loop domains in interphase *Xenopus* chromosomes is shorter in early embryos than in larval or adult cells.

THE SEQUENCE SPECIFICITY OF DNA REPLICATION IN *XENOPUS* EGGS

Unfertilized eggs of *Xenopus laevis* replicate injected DNA semiconservatively. This makes it possible to address the question posed above by asking what DNA sequences are necessary for initiation of replication in *Xenopus* eggs. Initial attempts to identify functional replication origins in this way produced conflicting results. On the one hand Harland & Laskey (1980) and McTiernan & Stambrook (1980) reported that initiation does not require specific DNA sequences. In contrast Watanabe & Taylor (1980) and subsequently Chambers, Watanabe & Taylor (1982) and Hines & Benbow (1982) reported that replication in *Xenopus* eggs involves specific DNA sequences. More recently Mechali & Kearsey (1984) have re-examined these reports and they have been unable to find evidence for preferential replication of any DNA sequences including those which had been reported to replicate preferentially, or including sequences selected from the *Xenopus* genome which replicate preferentially in yeast. Instead Mechali & Kearsey (1984) found that the efficiency of replication was related only to the size of the DNA template and not to its sequence.

These experiments cannot exclude the possibility that replication of the egg's chromosomal DNA depends on a sequence preference which is not revealed by injecting exogenous DNA templates. However they do allow two important conclusions. First they show that the enzymes of DNA replication in a *Xenopus* egg do not require a specialized DNA sequence to initiate efficient semiconservative replication. Second they show that a specific replication origin is not required for the regulatory mechanism which coordinates multiple initiations on a DNA molecule by preventing reinitiation within a single cell cycle. Since these are the two most obvious functions which a specific replication origin could perform, we are left with the problem of explaining why replication is ever sequence specific (Laskey & Harland, 1981), a point which is considered further below.

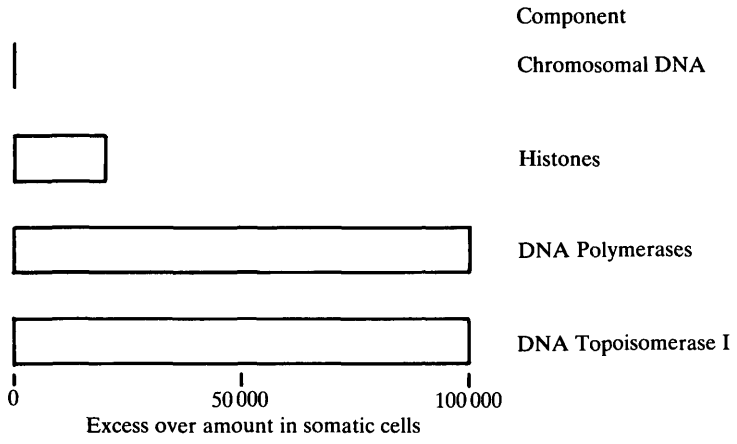

Fig. 3. Amounts of materials involved in chromosomes replication accumulated in eggs of *Xenopus laevis*.

THE IMPORTANCE OF PREFABRICATION IN ACHIEVING RAPID RATES OF CHROMOSOME REPLICATION

Initiation of replication at frequent intervals on DNA in early embryos is complemented by provision of a large maternal stockpile of materials involved in chromosome replication. For example DNA polymerase activities exceed the amounts in somatic cells by about 10^5 (Benbow, Pestell & Ford, 1975) and deoxynucleoside triphosphate pools are sufficient to synthesize 2500 diploid nuclei (Woodland & Pestell, 1972). As shown in Fig. 3, oogenesis also provides a stockpile of materials involved in other aspects of chromosome replication. The most thoroughly studied of these are the histones. Woodland & Adamson (1977) have shown that histone synthesis is uncoupled from DNA replication during oogenesis so that a maternal histone pool sufficient for about 20000 diploid nuclei is accumulated. Thus in *Xenopus* the problem of matching the rate of histone synthesis to DNA replication is overcome by providing preformed pools of both histones and histone mRNA. It has recently become clear that the stoichiometries of the stored histones differ markedly from those in chromatin. Thus histones H3 and H4 are present in a substantial excess over histones H2A and H2B (Kleinschmidt *et al.* 1985). The amount of histone H1 remains to be determined. Another interesting deviation from the stoichiometry in chromatin is seen for chromosomal protein HMG1. Although chromatin contains about ten times more of each histone than HMG1, the oocyte contains approximately equal amounts (Kleinschmidt *et al.* 1983).

THE CAPACITY FOR RAPID CHROMATIN ASSEMBLY IN *XENOPUS* EMBRYOS

Rapid cell division requires not just accelerated DNA replication but rapid replication of the entire chromosome structure. Condensed mitotic chromosomes

are seen every 35 mins during *Xenopus* cleavage. As seen in the preceding section a pool of histones is synthesized before this phase of rapid DNA synthesis and it would be enough to assemble at least 20 000 diploid nuclei if the histones were all present in equal amounts. The capacity for nucleosome assembly can also be titrated by microinjection or by incubating DNA in a homogenate of *Xenopus* eggs (Laskey, Mills, & Morris, 1977). This results in rapid assembly of the DNA into regularly spaced nucleosomes and allows an independent assay of the capacity for nucleosome assembly and of the minimum size of the histone pool. Note that this assay measures the amount of the least abundant core histone (therefore effectively H2A and H2B) rather than the average value reported by Woodland & Adamson (1977). This estimate shows that the histone pool is sufficient to assemble at least 7000 to 13 000 diploid nuclei (Laskey, Mills & Morris 1977; Laskey, Honda, Mills & Finch, 1978*a*). This estimate has been confirmed recently using homogenates of oocytes rather than eggs (Glikin, Ruberti & Worcel, 1984). Perhaps the most remarkable feature of this system is the speed of assembly. The homogenate from each mononucleate egg can assemble DNA equivalent to 7000–13 000 diploid nuclei into nucleosomes in only one hour.

ASSEMBLY FACTORS WHICH FACILITATE NUCLEOSOME ASSEMBLY FROM HISTONES AND DNA IN *XENOPUS* EGGS

Under certain conditions histones and DNA can self-assemble to form nucleosome cores (reviewed by Laskey & Earnshaw, 1980). Initially this was achieved by mixing in 2 M-NaCl and gradually dialysing against a series of changes of decreasing ionic strength. More recently methods have been described which allow assembly at physiological ionic strength in the absence of assembly factors. Thus Stein, Whitlock & Bina (1979) found that the precipitate which forms when DNA is mixed with histones can slowly redissolve over several hours forming nucleosome cores. Similarly Ruiz-Carillo, Jorcano, Eder & Lurtz (1979) showed that nucleosome cores can form when preformed histone dimers and tetramers are pumped slowly into an excess of DNA. Both methods clearly confirm that sufficient spatial information for nucleosome assembly exists in histones and DNA themselves, but neither can account for nucleosome assembly in a *Xenopus* egg, because both procedures are inhibited when histones are present in excess over DNA, yet *Xenopus* eggs contain a several thousand-fold excess of histones over DNA.

The efficiency of nucleosome assembly in *Xenopus* eggs suggested that assembly is mediated by assembly factors which facilitate interaction of histones with DNA (Laskey *et al.* 1978*b*). Fractionation of extracts revealed that histones co-fractionated with an assembly activity as negatively charged complexes (Laskey *et al.* 1978*a,b*). The assembly activity was negatively charged, resistant to nucleases, and slightly, but significantly, sensitive to proteases. This led us to propose that nucleosomes are assembled by an acidic protein which binds histones and transfers them to DNA (Laskey *et al.* 1978*a*). The activity was heat stable which greatly

simplified purification since most other proteins are precipitated by boiling and can therefore be removed by centrifugation. Resistance to heat precipitation, discrete size and highly acidic charge allowed rapid purification of a protein 'nucleoplasmin' with the properties predicted by the model. It consists of five subunits of about 30 000 molecular weight and isoelectric point 4·5–5·5 (Earnshaw, Honda, Laskey & Thomas, 1980; Mills, Laskey, Black & de Robertis, 1980). *In vitro* nucleoplasmin binds histones to form negatively charged complexes which can then interact with DNA to form nucleosome cores (Laskey *et al.* 1978*a*; Earnshaw *et al.* 1980). After nucleoplasmin was purified from total cell homogenates, it became clear that it is the most abundant protein in *Xenopus* oocyte nuclei occurring at 5–8 mg ml^{-1} in the nucleoplasm and forming 8–10 % of the total nuclear protein (Mills, *et al.* 1980; Krohne & Franke, 1980*a*).

Although nucleoplasmin clearly assembles nucleosome cores *in vitro*, until recently it has not been clear that it performs this role *in vivo*. Doubt arose initially because of a report that DNA topoisomerase I also acts as an assembly factor *in vitro*, but by interacting with DNA rather than with histones (Germond *et al.* 1979). However a subsequent report from the same laboratory has reinterpreted this claim (Nelson, Wiegand & Brutlag, 1981). A more serious doubt on nucleoplasmin's role *in vivo* arose from reports by Krohne & Franke (1980*a*,*b*) that they could not detect complexes between nucleoplasmin and histones in *Xenopus* oocytes using an anti-nucleoplasmin antiserum. Furthermore Kleinschmidt & Franke (1982) demonstrated definitively that histones H3 and H4 are complexed to two other acidic proteins of 100 000 molecular weight called N1 and N2. These proteins were discovered (Bonner 1975) in the same room as our early work on nucleosome assembly. Therefore we had expected them to be responsible for the nucleosome assembly activity, but had consistently found that they fractionated away from the major assembly activity (e.g. fig. 1 in Laskey *et al.* 1978*a*). In contrast activity cofractionated with nucleoplasmin, together with sufficient endogenous histones for assembly.

This paradox has been largely resolved by Kleinschmidt *et al.* (1985) who have established that both classes of complex coexist in *Xenopus* oocytes. One class of complex contains histones H3 and H4 bound to N1 and N2 while a second contains H3, H4, H2A and H2B bound to nucleoplasmin. Thus it appears that the excess H3 and H4 are bound to different binding proteins from the stoichiometric amounts of the four core histones. The exact stoichiometries in the complexes and the functional relationship between the two classes of complex remain to be discovered.

THE RELATIONSHIP OF DNA REPLICATION TO TRANSCRIPTION IN EARLY EMBRYOS

The early period of rapid cell division in both *Xenopus* and *Drosophila* embryos is characterized by relative transcriptional quiescence. In both cases transcription switches on suddenly at the exact time when the cell cycle elongates (McKnight &

Miller, 1976; Newport & Kirschner, 1982). It would be particularly interesting to know if strict sequence specificity of DNA replication is imposed at this point. Clearly this would be consistent with the increase in cell cycle time, but it might also offer an alternative explanation of the role of replication origins. As explained above, injection experiments with *Xenopus* eggs indicate that specific replication origins are not required for the replication enzymes to initiate or for the mechanism which co-ordinates multiple initiations by preventing reinitiation within a single cell cycle. An alternative attractive hypothesis is that they are required to define the boundaries of transcription units, so that a transcription unit is treated as a single unit for replication and chromatin assembly. There is abundant evidence that active genes are held in a different chromatin conformation from bulk chromatin (Weisbrod, 1982). Defining the sites at which replication, and hence chromatin assembly, start might ensure that entire transcription units are always assembled into the same type of chromatin structure. If so, then this level of regulation would be unnecessary in early embryos since they are transcriptionally quiescent. It will be interesting to test this correlation further by seeking sequence specificity of replication after the midblastula transition when chromatin replication slows down and gene expression is activated.

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DISCUSSION

Speaker: R. Laskey

Question from P. Hausen (Tubingen):
Does nucleoplasmin occur in somatic cells?

Answer:
Krohne & Franke [*Expl Cell Res.* **129**, 167–189 (1980)] argued that it was present in a wide range of somatic cells. In *Xenopus* cultured cells my colleague Stephen Dilworth has evidence from studies with monoclonals that nucleoplasmin is present, but not in anything like the amounts that are found in oocytes.

Question from C. Wylie (St. Georges, London):
I missed some of the things you said about the structure of the nucleoplasmin. Does each molecule have 5 binding sites?
Answer:
It's a pentamer of 5×30000 molecular weight and each pentamer has a polyglutamic acid tract of about 16 glutamic acids in a row which is punctuated in one of the polypeptides and not in the other (C. Dingwall, S. Kearsey, S. Black and T. Bürglin, unpublished). So, it's hard to see how you can build an effective binding site for each polypeptide, whereas when you put these together you would have a very effective binding site. We know it is a pentamerically symmetrical molecule: in the EM you can actually see 5 arms. We know that polyglutamic-acid-containing stretches can be cleaved off preferentially by partial proteolysis so they must be exposed. There are not many hydrophobic residues with the exception of alanine in the tails, though we know the core is rich in hydrophobics. It is tempting to think that the tails may be flexible and able literally to wrap around the histones and DNA. That's something that could be tested by NMR – but it hasn't been done yet.

Question from I. Dawid (NIH, Bethesda):
What is known in other animals, for example, *Drosophila* eggs, which also replicate their DNA pretty fast – do they have nucleoplasmin-like molecules?

Answer:
I think there is evidence that *Drosophila* cells do contain nucleoplasmin, but it is weak and indirect and has never been published, it dates from experiments with Krohne and Franke's original antiserum. I can't tell you about other non-amphibian species though Krohne and Franke would argue that it is widespread, but I can say that there appears to be an almost identical protein as quite an abundant component of wheatgerm. This is the work of Byron Lane in Toronto. The protein has several odd properties like unusual stability to boiling in SDS. It remains pentameric on

SDS gels, unless it is boiled vigorously at low ionic strength when it dissociates just like the frog protein. The wheatgerm protein is totally resistant to pepsin, whereas pepsin makes a unique cleavage in the *Xenopus* protein and in fact the wheatgerm protein can be purified by incubating total wheatgerm with pepsin and keeping the one uncut protein that's left.

We're actually investigating the possibility of wheatgerm as a bulk source of nucleoplasmin. So, if it is in wheatgerm, I'd be willing to bet it will turn up elsewhere as well, but only in small amounts.