Cyclin B mRNA depletion only transiently inhibits the *Xenopus* embryonic cell cycle

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Summary

The control of the cell cycle is dependent on the ability to synthesize and degrade proteins called cyclins. When antisense oligonucleotides are used to deplete *Xenopus* embryos of mRNA encoding cyclin B protein, embryonic cleavage is inhibited. Surprisingly, after missing several rounds of cleavage, the cell cycle and cell division resumes. These studies indicate that the early embryonic cell cycle can proceed with undetectable levels of cyclin B encoding mRNA. In contrast, other events of normal development, including the activation of embryonic transcription and gastrulation, are inhibited.

Key words: cyclin, cell cycle, antisense, *Xenopus*, mRNA.

Introduction

The maturation of *Xenopus* oocytes and the early rounds of embryonic division depend on the presence of an active MPF (maturation promotion factor) complex (Murray and Kirschner, 1989; Dunphy *et al.* 1988; Lohka *et al.* 1988; Draetta *et al.* 1989; Labbe *et al.* 1989; Gautier *et al.* 1990). Active MPF consists of a complex of histone H1 kinase activity, now commonly referred to as *cdc2* kinase and cyclin B. In oocytes and early embryos, there are two different cyclin B encoding mRNAs present that are translated to produce similar proteins, cyclin B1 and cyclin B2 (Minshull *et al.* 1989). Synthesis of cyclin B protein is correlated with entry into the M phase of the cell cycle, and the degradation of cyclin B is required for the exit out of the M phase (Murray and Kirschner, 1989; Dunphy *et al.* 1988; Lohka *et al.* 1988; Draetta *et al.* 1989; Labbe *et al.* 1989; Gautier *et al.* 1990). *Xenopus* egg extracts have been prepared that support multiple rounds of the cell cycle and DNA replication *in vitro*. If these extracts are depleted of both cyclin B encoding mRNAs, they fail to cycle. Readdition of either cyclin B encoding mRNA or cyclin B protein can restore activity indicating that the production of cyclin B1 or B2 is essential for *in vitro* progression through the cell cycle (Minshull *et al.* 1989; Murray and Kirschner, 1989b; Murray *et al.* 1989).

The role of cyclin proteins during the embryonic cell cycle has been difficult to study *in vivo*. We have developed a strategy based on the embryonic injection of modified antisense oligodeoxyribonucleotides (ODNs) to examine the developmental importance of cyclin B protein. Unmodified ODNs that are injected into developing *Xenopus* embryos are rapidly degraded making *in vivo* inhibition of the production of a specific protein difficult (Woolf *et al.* 1990; Dagle *et al.* 1990). Recently we described a class of modified ODNs that are resistant to degradation by the most active nucleases found in *Xenopus* embryos. The ODN modification still allows hybridization to specific mRNAs and subsequent cleavage by endogenous RNAase H (Dagle *et al.* 1990). The result is the selective degradation of the targeted mRNA. The injection of modified ODNs complementary to cyclin B mRNA into one cell of a two-cell embryo leads to aberrant development of the injected cell (Dagle *et al.* 1990, 1991). Because cyclin protein is degraded as cells exit the M phase of the cell cycle, targeted degradation of cyclin B mRNA should rapidly alter the availability of cyclin B protein. Using this approach, we present data suggesting that the early embryonic cell cycle can proceed with undetectable levels of cyclin B mRNA.

Materials and methods

Materials

2-Methoxyethylamine was purchased from Aldrich and stored over 30 nm molecular sieves. Human chorionic gonadotropin, cysteine and Ficoll-400 were purchased from Sigma. Klenow and nucleoside triphosphates were purchased from Promega. All radioactive nucleotides were purchased from Amersham. *Xenopus laevis* were purchased from *Xenopus* I (Ann Arbor, MI).

Synthesis of modified oligonucleotides

Synthetic oligonucleotides were made on an ABI Model 381A DNA synthesizer using hydrogen phosphonate chemistry as previously described (Dagle *et al.* 1990). Briefly, coupling and oxidation were performed according to the manufacturer's recommendations. Oxidative amidation was performed by treating hydrogen phosphonate diesters with 10% 2-methoxyethylamine in carbon tetrachloride (Froehler, 1986). The reaction volume was 3.3 ml and the reaction time was one h. Oligonucleotides were deblocked and removed from the controlled-pore glass support with a 6 h incubation in ammonium hydroxide at 60°C. DMT-protected oligonucleotides were purified by reversed-phase HPLC, detritylated and again purified by HPLC. To desalt and remove any trace organic contaminants, the oligonucleotides were passed through a NAP-5 gel filtration column (Pharmacia) twice. The oligonucleotides were dried under vacuum and resuspended in sterile water.

Microinjection of embryos

Eggs were obtained and fertilized as previously described (Rebagliati *et al.* 1985). Aliquots of oligonucleotides were injected into the cytoplasm of embryos using the technique described by Colman, 1984. Fertilized eggs were dejellied for 10–15 min in 2% cysteine, 0.1×MBSH. The eggs were then rinsed and placed in 0.1×MBSH containing 5% Ficoll-400 prior to injection. Injection volume was 10 nl, and approximately 5 ng of ODN were injected into each embryo just prior to first cleavage. Morphological studies were repeated more than 5 times with more than 30 embryos per sample, RNA analysis 3 times, and [³H]thymidine incorporation 3 times. Representative data are shown.

During the course of these and other experiments, we have injected in excess of twenty different individual synthetic batches of ODN. The sequences injected contain a variety of base compositions including GTAACTAGCCTGGAA (A:5,C:3,T:3,G:4), TTCACCCATAGTAAC (A:5,C:5,T:4, G:1), ATCCAAATTCTGGGA (A:5,C:3,T:4,G:3), GGGC-AACAGCTCCAT (A:4,C:5,T:2,G:4), ATGGTGCACCT-GACT (A:3,C:7,T:4,G:4), CCCTATCCGATCCATT (A:3, C:7,T:5,G:1), CTGACAACATGACTGC (A:5,C:5,T:3, G:3), GTCTCAGCCACACCATA (A:5,C:7,T:3,G:2), ATA-AGGTTCTACAGGT (A:5,C:2,T:5,G:4), TTCACCCATA-GTAAC (A:5,C:5,T:4,G:1). We have observed a number of different effects caused by the introduction of ODN, but also have seen perturbations due to the introduction of a microinjection needle. For instance, some batches of embryos show a modest retardation of division rate even when injected with water (see for instance the slight delay in the onset of embryonic transcription in water-injected embryos in Fig. 1B). Occasionally we have observed the irreversible arrest of division that coincides with the site of injection. We also note, as have others (Woolf *et al.* 1990; Smith *et al.* 1990), that the introduction of greater than 20 ng of ODN/embryo often leads to non-specific toxicity. On balance, we observe that some batches of embryos do not respond well to injection, and find water injection controls as well as injection controls with other modified ODNs useful in uncovering non-specific effects due to injection. We also observe that the degradation of some maternal mRNA sequences does not lead to a discernable phenotype, or prevent the normal development of the embryo into a tadpole.

Analysis of RNA

Embryos were frozen in dry ice prior to processing. Embryo RNA was extracted as previously described (Dagle *et al.* 1990)

and separated by size by electrophoresis through a formaldehyde/agarose gel prior to transfer to Nytran (Schleicher and Schuell) filters as described in Rebagliati *et al.* 1985. End-labeled [³²P]oligonucleotides specific for either cyclin B1 (nucleotide 120–170, Minshull *et al.* 1989) or cyclin B2 (nucleotide 53–110, Minshull *et al.* 1989) were used as hybridization probes to detect cyclin B1 or B2 mRNA. The GS17 probe used was a random primed (³²P-labeled) *Sma*I to *Eco*RI GS17 cDNA fragment (Krieg and Melton, 1985) and the cyclin A probe used was a random primed (³²P-labeled) *Eco*RI cyclin A cDNA fragment (Minshull *et al.* 1990). After hybridization and washing, filters were exposed at −70°C to Kodak X-OMAT AR film using an intensifying screen.
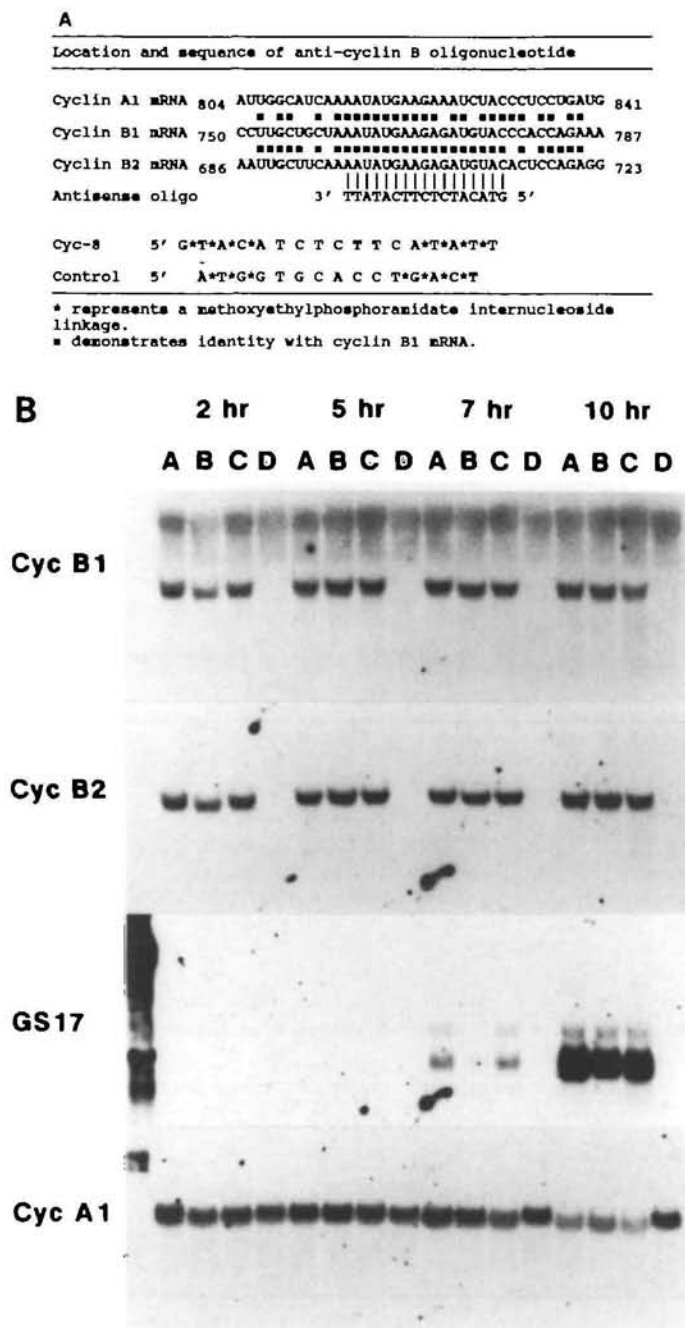
Analysis of DNA synthesis

DNA synthesis in embryos was monitored by continuously labeling in a solution of 900 μCi ml⁻¹ [³H]thymidine 0.1× MBSH containing 5% Ficoll-400 by a procedure slightly modified from (Newport and Kirschner, 1982a). Five embryos were assayed for each time point. After washing in water (500 times the labeling volume) the embryos were lysed in 200 μl of 1% SDS, 10 mM Tris (pH 7.0) and 10 mM EDTA by moving the resuspended embryos vigorously in and out of a 200 μl pipet tip. 10 μg of proteinase K were added to the solution, followed by an incubation at 37° for one hour. After two phenol extractions and one ether extraction, the DNA was ethanol precipitated, and the pellets resuspended in 200 μl of 10 mM Tris (pH 7.0) and 1 mM EDTA. 25 μl of 100% TCA were added to the solution which was then chilled on ice for 30 min and filtered through Whatman GFA filters. The filters were washed individually three times with 4 ml of ice-cold 9% TCA, and once with 4 ml ice-cold ethanol, allowed to air dry and counted in the presence of 5 ml Budget-solve (RPI).

Results

Cyclin B1 and B2 mRNA depletion transiently inhibits cell division and DNA synthesis

The cyclin B encoding mRNAs share a common region of nucleotide sequence (Minshull *et al.* 1989), which allows us to target both messages for degradation with one ODN. Injection of 5 ng of the modified ODN, cyc-8 (Fig. 1A) results in the degradation of both cyclin B1 and cyclin B2 mRNAs within two hours (Fig. 1B). Coincident with the reduction of cyclin B1 and B2 mRNA to undetectable levels, is the arrest of early cleavage (Fig. 2, 2 h pictures). Two hours after cyc-8 injection the embryo had divided three times, but arrested at the eight-cell stage while control embryos continued with fourth and fifth cleavages. Surprisingly, after missing several rounds of cleavage, cell division resumes (Fig. 2, 2.5 h post-injection), with a new division every 30 to 40 min, until cyc-8 injected embryos have about the cell number normally seen at the midblastula stage (about 4000 cells, compare 5.5 h controls with 10 h cyc-8 injected). Cellular division again arrests, about 7 h after injection (7 h cyc-8 embryos appear very similar to 10 h cyc-8 injected embryos), and cyc-8 injected embryos do not gastrulate.

Several parameters were examined in an effort to understand the resumption of cell division following cyc-8 mediated arrest. Embryonic DNA synthesis was

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A

Location and sequence of anti-cyclin B oligonucleotide

Cyclin A1 mRNA 804 AUUGGCAUCAAAAUAUGAAGAAAUCUACCCUCCUGAUG 841
Cyclin B1 mRNA 750 CCUUGCUGCUAAAUAUGAAGAGAUGUACCCACCAGAAA 787
Cyclin B2 mRNA 686 AAUUGCUUCAAAAUAUGAAGAGAUGUACACUCCAGAGG 723

Antisense oligo 3' TTATACTTCTCTACATG 5'

Cyc-8   5' G*T*A*C*A T C T C T T C A*T*A*T*T

Control 5' A*T*G*G T G C A C C T*G*A*C*T

* represents a methoxyethylphosphoramidate internucleoside
linkage.
■ demonstrates identity with cyclin B1 mRNA.

B

	2 hr	5 hr	7 hr	10 hr
	A B C D	A B C D	A B C D	A B C D

Cyc B1

Cyc B2

GS17

Cyc A1

Fig. 1. (A) The homologous regions of the maternal cyclin B mRNAs in *Xenopus* and the sequence of the modified ODN (cyc-8) used for the targeted degradation of cyclin B messages. The sequence for *Xenopus* cyclin A is also presented. Modified linkages were methoxyethylphosphoramidates and are indicated by *. (B) The levels of selected mRNA during embryogenesis. RNA was extracted from 5 embryos at 2, 4, 7, and 10 h after injection. For each time point, embryos were harvested that were: A, non-injected; B, water injected; C, injected with a modified but unrelated nucleotide sequence; or D, injected with a modified ODN directed against cyclin B (cyc-8). RNA was separated by size on a denaturing gel and transferred onto a nylon filter. Sequential hybridization of radioactive probes was used to determine the level of cyclin B1, cyclin B2, GS17 and cyclin A mRNA in each lane. End labeled (^{32}P) oligonucleotides specific for either cyclin B1 or cyclin B2 were used as hybridization probes to detect cyclin B1 or B2 mRNA. The GS17 probe used was a random primed (^{32}P-labeled) *Sma*I to *Eco*RI GS17 cDNA fragment (Krieg and Melton, 1985) and the cyclin A probe used was a random primed (^{32}P-labeled) *Eco*RI cyclin A cDNA fragment (Minshull *et al.* 1990).

bryos (Dagle *et al.* 1990, 1991). *De novo* synthesis of cyclin B mRNA might permit the embryos to overcome the initial block of replication. This seemed unlikely because *Xenopus* embryos do not normally begin transcription until the 4000-cell stage (Newport and Kirschner, 1982b), commonly referred to as the midblastula transition (MBT). We were not able to detect cyclin B mRNA replacement even 10 h after cyc-8 injection (Fig. 1B). Embryonic activation of transcription was also monitored by measuring levels of GS17 mRNA, one of the earliest mRNAs to appear during the MBT (Krieg and Melton, 1985). GS17 transcription begins on schedule in both the uninjected and control injected embryos, but is not detectable after treatment with cyc-8 (Fig. 1B) even many hours after the normal onset of embryonic transcription. The onset of transcription of GS17 in water-injected controls was slightly later than in non-injected or control oligo-injected embryos (Fig. 1B, 7h samples) but transcription and development seemed to be restored by 10h after injection. This delay was not seen in other trials.

The resumption of early development in the cyc-8 injected embryos neither depends upon nor includes the activation of normal embryonic transcription.

Cyclin A encoding mRNA is present but does not prevent the transient inhibition of cell division or later defects

Recent reports indicate the presence of a maternal cyclin A mRNA in *Xenopus* that is translated at lower rates than cyclin B (Minshull *et al.* 1990). In *Xenopus*, cyclin A, like cyclin B protein, has been recovered complexed to the *cdc2* kinase portion of MPF. Cyclin A has been examined in clam (Swenson *et al.* 1986) and in fruit fly (Whitfield *et al.* 1989; Lehner and O'Farrell, 1989; Lehner and O'Farrell, 1990) and is able to carry out several of the functions of cyclin B, although
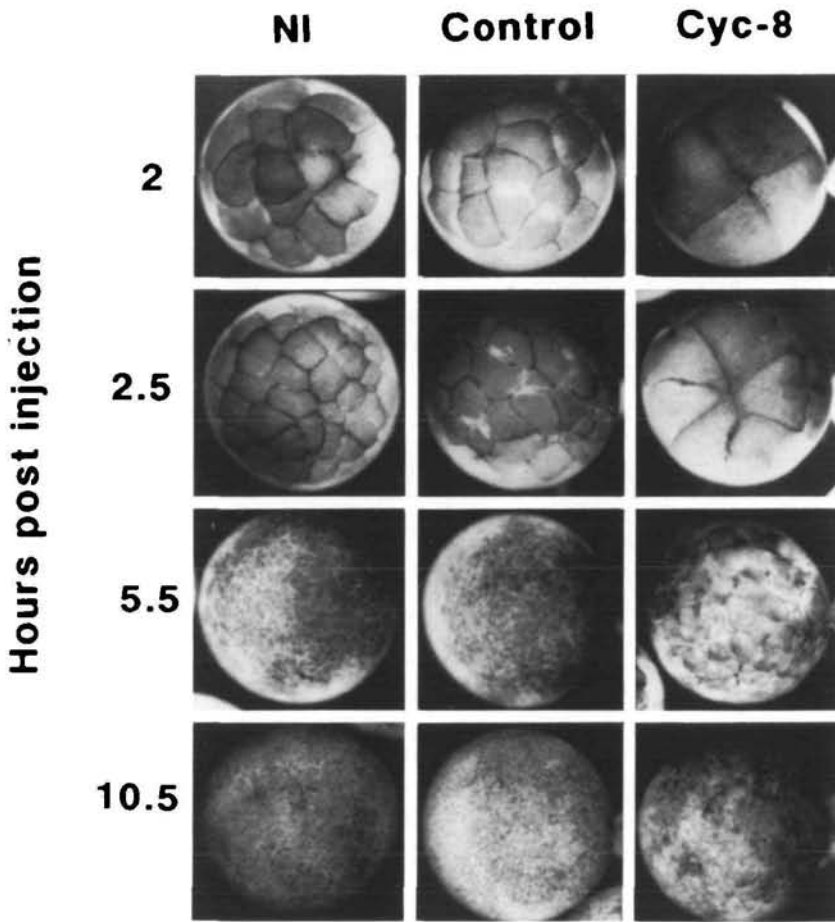
examined by measuring [^{3}H]thymidine incorporation after ODN injection (Fig. 3). Control embryos, uninjected or injected with a similarly modified ODN of an unrelated sequence, were also examined. While thymidine incorporation increased steadily in both control groups, it is delayed in cyc-8 injected embryos; however, thymidine incorporation resumed between 2 and 3 h after injection indicating that DNA replication, like cell division, was no longer blocked.

Cyclin B1 and B2 mRNA depletion affects normal onset of transcription

Although modified ODNs are substantially more stable than their unmodified counterparts, they can be degraded by endonucleases present in *Xenopus* em-

NI   Control   Cyc-8

Hours post injection

2

2.5

5.5

10.5

Fig. 2. Morphology of non-injected (NI), control ODN (control) and cyc-8 injected embryos at 2, 2.5, 5.5 and 10.5h after injection. Embryos are oriented so that the *injection site is on the right of the* photograph.

differences in activity and in temporal expression have been noted (Westendorf *et al.* 1989; Lehner and O'Farrell, 1989; Lehner and O'Farrell, 1990; Minshull *et al.* 1990). The cyc-8 ODN used to target cyclin B mRNA degradation contains substantial complementarity to *Xenopus* cyclin A, with only two bases mismatched (indicated in Fig. 1A). Only one of the

Fig. 3. The effect of cyc-8 injection on DNA synthesis. Embryos were dejellied with 2% cysteine incubated in 900 μCi ml^{-1} [^{3}H]thymidine. Five embryos were assayed for each time point. ▲ – control ODN injected, ● – no injection, ■ – cyc-8 injected.

mismatches is in the region of the ODN that would serve as a substrate for RNAase H, where the cyclin B mRNAs contain a guanosine the cyclin A mRNA has an adenosine. Despite this large degree of complementarity, there is not a detectable effect of cyc-8 on the cyclin A mRNA pool (Fig. 1B) at any of the timepoints examined, indicating the high level of specificity that can be obtained using modified ODNs to degrade an endogenous mRNA. The level of cyclin A mRNA in cyc-8 injected embryos does not decrease 10h after injection, in contrast to the reduction of this mRNA found in control embryos. We conclude that cyclin A alone is not enough to prevent the transient inhibition of the early embryonic cell cycle or the block that occurs later. Cyclin A may however, be responsible for the resumption of cleavage.
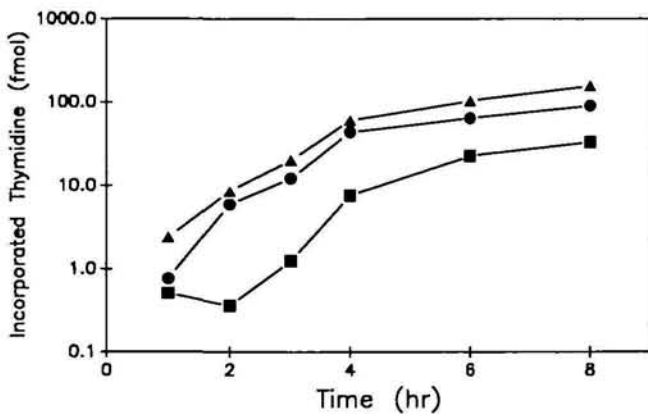
Discussion

The control of the cell cycle during early development requires the rapid implementation of several different strategies. In *Xenopus*, at least three different control points can be recognized, the release of the arrest of meiosis found in late stage oocytes, the rapid DNA synthesis and synchronized cell division characteristic of the first twelve divisions, and finally a shift to a slower cell cycle, when cell division is no longer synchronized, and embryonic transcription begins. Experiments using

the release of meiosis and oocyte maturation (Murray and Kirschner, 1989; Dunphy *et al.* 1988; Lohka *et al.* 1988; Draetta *et al.* 1989; Labbe *et al.* 1989; Gautier *et al.* 1990), and *in vitro* egg extracts that can mimic the early cell cycle have provided evidence that an active MPF, consisting of cdc2 kinase and cyclin B is the regulator of the cell cycle. Both in cycling extracts (Minshull *et al.* 1989; Murray and Kirschner, 1989*b*; Murray *et al.* 1989) and as presented here, *in vivo*, reduction of the maternal pools of cyclin B1 and B2 mRNA lead to an arrest of the cell cycle. However, unlike observations made using *in vitro* cell cycling extracts, the *in vivo* arrest can be overcome and cell division and DNA replication restored.

The reactivation of the cell cycle occurs well before the normal onset of embryonic transcription, and in the absence of detectable cyclin B mRNA. Therefore, either other maternal mRNA(s), perhaps that encoding cyclin A, or alternatively maternal proteins as yet uncharacterized in *Xenopus*, are responsible for the resumption of cell division. Cyclin A mRNA levels are constant in the studies described here, but they do not prevent the initial inhibition of cell division, suggesting that cyclin B may have a special role to play during the time period of the first few divisions. The transient nature of the cyc-8 mediated inhibition suggests the delayed activation of a translational or post-translational regulator of the embryonic cell cycle.

In vitro, the rate of progression through the cell cycle may be linked to the rate of cyclin B protein synthesis (Murray and Kirschner, 1989*a*). After depletion of the endogenous pool of cyclin B encoding mRNA, the accumulation of new cyclin B protein derived from exogenously added cyclin B mRNA correlated to the length of interphase. The experiments presented here indicate that even with undetectable levels of cyclin B mRNA, that progression through the cell cycle (once it resumes) does not dramatically differ from what is normally seen. Recent experiments (Solomon *et al.* 1990) suggest that there is a threshold level of cyclin B protein required for activation of the MPF complex. If the threshold model is correct perhaps the earliest divisions require more cyclin B protein than later cleavages, and that even a trace amount of cyclin B mRNA, not detectable by the methods used here, is sufficient to cause the cell cycle recovery that we observe.

Finally, although anti-cyclin B injected embryos do resume cell division they do not activate normal embryonic transcription, and do not gastrulate. This developmental defect persists even in the presence of the maternal complement of cyclin A encoding mRNA. Cell division stops for a second time when close to the normal number of cells required for the transition to a slower, non-synchronous cell cycle has been obtained. This indicates that the maternal pool of cyclin B encoding mRNA may be important for this transition to occur. The level of cyclin A mRNA is reduced in all the control embryos by 10 h after injection. The persistence of cyclin A mRNA in cyc-8 injected embryos may indicate that whatever mechanism is normally used to

degrade this mRNA is not activated. These findings are reminiscent of cell cycle mutant studies carried out in *Drosophila*, where different cyclin proteins were shown to have distinctive developmental roles (Lehner and O'Farrell, 1990). Our experiments differ from those in *Drosophila*, in that the mitotic cycle continues in *Xenopus* in the apparent absence of cyclin B.

The results presented here use the injection of modified ODNs to specifically degrade cyclin B mRNA during early development. The introduction of any foreign substance during this sensitive time runs the risk of causing non-specific defects. We previously described the use of such ODNs and characterized their stability and effectiveness during embryogenesis (Dagle *et al.* 1990, 1991). We have injected a variety of DNA sequences, both modified and unmodified, and at the concentrations described in this study have never seen a similar phenotype, that is, transient arrest of early cleavage, followed by resumption of the early cell cycle (Dagle *et al.* 1990, 1991; Dagle and Weeks, (unpublished)). We are continuing these studies to examine if cyclin B1 and B2 have different roles *in vivo*, and to further examine the role cyclin A plays during early development in *Xenopus*.

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