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Protein translation during early cell divisions of sea urchin embryos regulated at the level of polypeptide chain elongation and highly sensitive to natural polyamines

Annabelle Monnier, Julia Morales, Patrick Cormier, Sandrine Boulben, Robert Bellé and Odile Mulner-Lorillon

Station Biologique de Roscoff, Université Pierre et Marie Curie (UFR 937), Centre National de la Recherche Scientifique (CNRS, UPR 9042), BP 74, 29682 Roscoff Cedex, France

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Summary

Protein synthesis was analysed following fertilisation in sea urchin. Fluctuations in the accumulation of neo-synthesised proteins were observed during the first cell cycles. Accurate translation analyses were performed from lysates prepared from early embryos. The lysates readily translated endogenous pre-initiated mRNAs allowing the determination of elongation rates in the absence of re-initiation *in vitro*. The translation capacity of embryo lysates increased 18-fold from 0 to 90 min after fertilisation, reflecting the increase in the amount of pre-initiated mRNAs during early development. Kinetics analysis at a short time interval during the course of early development (240 min) showed an overall increase in the elongation rate (> 10-fold) which is regulated by pauses in synchrony with the cell divisions. Elongation activity in the lysates was highly sensitive to the natural polyamines, spermine (ID_{50} = 0.2 mM) and spermidine (ID_{50} = 1.8 mM), indicating high potential regulation by the intracellular level of polyamines in embryos. The regulation in the elongation changes associated with the early embryo cell divisions is discussed in the light of the physiological fluctuations in polyamine concentrations.

Keywords: Cell-free translation, Polyamines, Polypeptide elongation, Protein translation, Sea urchin early development

Introduction

Fertilisation of sea urchin oocytes has been associated with an increase in the translation efficiency of the embryos, which is necessary for early development (Epel, 1967, 1990). The change has been demonstrated to result from an increase in the initiation rate of the large pool of masked maternal mRNAs (Humphreys, 1971; Grainger & Winkler, 1987) together with an increase in the elongation rate (Brandis & Raff, 1979; Hille & Albers, 1979). Cell-free systems have contributed greatly to the understanding of the molecular mechanisms of translation (Merrick & Hershey, 1996) since they contain the different classes of factors for initiation, elongation and termination required for efficient translation (Merrick & Hershey, 1996). Most attention has focused on the mechanisms of initiation control (Gray & Wickens, 1998) and, for instance, on the role of the initiation factors eIF4E (McKendrick *et al*., 1999) and eIF2B (Kimball, 1999) and their multiple levels of regulation (Kimball, 1999; Raught & Gingras, 1999). The mechanisms responsible for the regulation of translation in embryos have mostly been investigated at the level of initiation (Winkler *et al*., 1985; Colin *et al*., 1987; Hansen *et al*., 1987; Lopo *et al*., 1989) and led to the evidence for a release of the inhibition of initiation factor eIF4E (Jagus *et al*., 1992, 1993). By contrast, elongation regulation has not been characterised.

Fertilisation initiates the synchronous cell divisions of early development (Epel, 1990). It has been well established that the M-phase of the cell cycle is associated with a general repression in translation activity (Kanki & Newport, 1991). Early development offers the opportunity to analyse both the increase in protein synthesis post-fertilisation, and the regulation of translation during the cell cycle. We report here accur-

All correspondence to: Dr Odile Mulner-Lorillon, Station Biologique de Roscoff BP 74, 29682 Roscoff Cedex, France. Tel: +33 (0) 2 98 29 23 37. Fax: +33 (0) 2 98 29 23 06. e-mail: mulner@sb-roscoff.fr

ate determination of translation changes during early development of the sea urchin *Sphaerechinus granularis*.

Among the many factors which could regulate translation, attention was focused on natural polyamines (Igarashi & Kashiwagi, 2000) for three reasons: the existence of physiological fluctuations in polyamine levels during early development (Kusunoki & Yasamasu, 1976), the reported sensitivity of translation in cell-free extracts to spermidine concentrations (Hunter *et al.*, 1977; Igarashi *et al.*, 1988) and the increasing evidence for involvement of polyamines in cell cycle regulation (Li *et al.*, 1999; Ray *et al.*, 1999; Alm *et al.*, 2000). We report a potent effect of polyamines on the elongation rate, discussed in the light of the translation changes observed during early development.

Material and methods

Handling of eggs and embryos

The *Sphaerechinus granularis* sea urchins, collected in the Brest area, were kept in running sea water. Spawning of eggs was induced by intra-coelomic injection of 0.1 M acetylcholine and eggs were collected in 0.22 µm Millipore-filtered sea water.

For *in vitro* fertilisation and culturing, eggs were suspended in Millipore-filtered sea water (10% suspension) containing 1 mM 3-amino-1,2,4-triazole and 0.1% glycine. Diluted sperm was added to the eggs and was withdrawn after the occurrence of fertilisation as judged by membrane elevation around the eggs. Experiments were performed only on batches exhibiting greater than 95% fertilisation. All the procedures were carried out at 15 °C.

In vitro protein labelling

Ten millilitres of unfertilised egg suspension (10%) was labelled for 2 h in Millipore-filtered sea water containing 50 µCi of L-[^{35}S]methionine (1000 Ci/mmol; Amersham France). Eggs were then harvested by centrifugation, rinsed three times, resuspended in Millipore-filtered sea water and fertilised as above. At different times, 1 ml aliquots were rapidly centrifuged and the eggs pellets were frozen. Packed embryos were suspended in 400 µl ice-cold buffer containing 60 mM β-glycerophosphate, 15 mM *p*-nitrophenyl phosphate, 25 mM 4-morpholinepropanesulphonic acid (MOPS), pH 7.2, 15 mM EGTA, 15 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM disodium phenylphosphate, 1 mM 4-(2-aminoethyl)-benzenesulphonyl fluoride (AEBSF), 10 µg/ml soybean trypsin inhibitor, 100 µM benzamidine, and homogenised through a 25 G needle. The homogenates were clarified by 13 000 r.p.m. centrifugation for 10 min in a Sorvall RMC 14. [^{35}S]methionine incorporation was measured on duplicate aliquots of the supernatants after 10% trichloroacetic acid (TCA) precipitation on Whatman 3M filters and counting in the presence of Optiphase Supermix scintillation liquid in a 1450 MicroBeta counter (Wallace, EG&G Instruments).

Assay of histone H1 kinase activity

Histone H1 kinase was purified from the above 13 000 r.p.m. supernatants by affinity chromatography on p13^{suc1}-Sepharose beads prepared in the laboratory as recommended by the manufacturer (Pharmacia). The supernatants were incubated for 30 min under constant rotation at 4 °C in the presence of 10 µl p13^{suc-1}-Sepharose beads. The bound kinase was assayed in 50 µl final volume incubation containing 1 µCi [γ-^{32}P]ATP (3000 Ci/mmol; Amersham France), 10 µM unlabelled ATP, 10 µg histone H1 (Type III-S, Sigma) in buffer containing 60 mM β-glycerophosphate, 30 mM *p*-nitrophenyl phosphate, 25 mM MOPS, pH 7.2, 5 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT and 0.1 mM sodium orthovanadatate. After 10 min at 30 °C, the reaction was stopped by chilling the tubes in ice. Duplicate aliquots of 5 µl were spotted on Whatman P81 phosphocellulose papers, washed five times in 1% phosphoric acid and counted in water by Cerenkov counting.

Preparation of the lysates

The lysates were prepared from fresh eggs or embryos by a modification of the protocol elaborated by Jagus and co-workers (Hansen *et al.*, 1987; Akkaraju *et al.*, 1991). Egg and embryo suspensions were briefly centrifuged and re-suspended in Millipore-filtered sea water acidified to pH 5 for 10 min to remove the jelly count. The cells were rinsed twice in ice-cold buffer A (105 mM K gluconate, 40 mM NaCl, 1 mM $Mg(CH_3COO)_2$, 1 mM spermidine, 1 mM DTT, 10 mM EGTA, 300 mM glycine, 220 mM glycerol, 50 mM Hepes, pH 7.2). After centrifugation, egg and embryo pellets were homogenised through a 25 G needle in an equal volume of buffer A supplemented with 300 U/ml ribonuclease inhibitor (Ambion), 1 mM AEBSF and 0.5 mM glucose-6-phosphate. The homogenates were centrifuged at 10 000 r.p.m. for 15 min at 4 °C in a Jouan AK 100–21 rotor. The supernatants were dialysed against buffer A without glycerol, adjusted to 2 mM EGTA and supplemented with 0.5 mM glucose-6-phosphate at 4 °C for 90 min, with buffer changes every 30 min. Aliquots were made and immediately frozen.

Depletion of endogenous mRNAs

Aliquots (100 µl) of lysate were incubated with 10 ng

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bovine pancreatic ribonuclease A (Boehringer) at 10 °C. After 20 min, 1 µl of 100 mM DTT and 40 units of ribonuclease inhibitor (Ambion) were added and incubation continued for 10 min. The lysate was then supplemented with 5 µg calf liver tRNA (Sigma) before use or freezing (Matthews & Colman, 1991).

Cell-free protein synthesis assay

All assays were performed with 0.8 volume of eggs or embryos lysate. Optimal final concentration conditions for the other components were: 2 units creatine phosphokinase (Sigma), 10 mM phosphocreatine, 1 mM ATP, 0.5 mM GTP, 100 µM amino acids minus methionine, 10 µCi or 0.2 µM of L-[^{35}S]methionine (specific activity 1000 Ci/mmol; Amersham France), 150 mM $K(CH_3COO)_2$ and 1 mM $Mg(CH_3COO)_2$. The translation assay was incubated at 20 °C for the desired time, as indicated. Translation activity was estimated on 5–10 µl aliquots spotted in triplicate on 3M Whatman filters. After 10% TCA precipitation, radioactivity in proteins was measured by scintillation counting (Clemens, 1984). Another 5–10 µl aliquot was treated for SDS-12% polyacrylamide gel electrophoresis (Laemmli, 1970). Qualitative analysis of the neo-synthesised polypeptides was performed by autoradiography on β-max Hyperfilm (Amersham).

Incubations with rabbit reticulocyte lysate (Ambion) were performed in the same conditions at a temperature of 30 °C.

RNA preparation

The capped mRNA transcripts from different DNA, *Xenopus* EF-1 A (control template from the Ambion kit), *Xenopus* EF-1 δ (Minella *et al*., 1996) and *Sphaerechinus* EF-1 δ (Delalande *et al*., 1998) were obtained by a standard transcription protocol (mMessage mMachine from Ambion).

Results

Protein synthesis following fertilisation

Protein synthesis, estimated as [^{35}S]methionine incorporation into proteins, was analysed in early embryos. As already reported (Epel, 1967, 1990), the amount of labelled proteins was found to increase greatly after fertilisation. Furthermore, using short interval time course determinations, fluctuations in the accumulation of neo-synthesised proteins were consistently observed during the first hours of development (Fig. 1). When cell cycles were monitored by histone H1 kinase activity of embryo extracts, protein accumulation was reproducibly found to slow down in synchrony with the cell cycle as judged from the histone H1 kinase activity measured in parallel (Fig. 1).

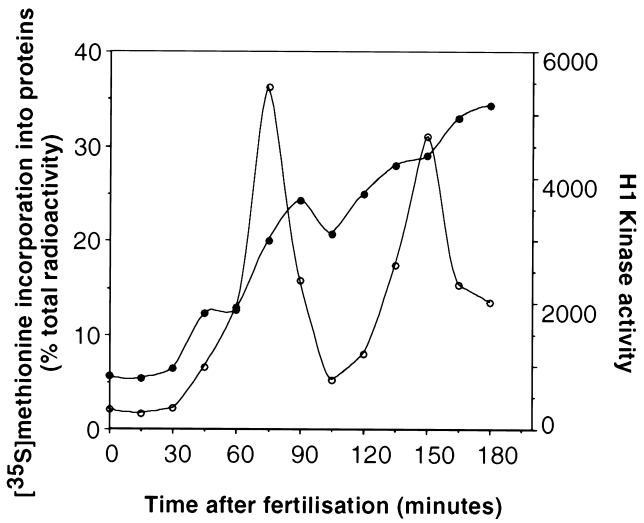

Figure 1 Protein synthesis and H1 kinase activity *in vivo*. Embryos were labelled in the presence of [^{35}S]methionine and processed for the determination of protein synthesis and histone H1 kinase activity at the indicated time after fertilisation. Filled circles, [^{35}S] methionine incorporation into proteins; open circles, H1 kinase activity in arbitrary units.

To characterise further the fluctuations observed in the accumulation of proteins during the first cell cycles, accurate translation determinations from embryo lysates were performed.

Translation determinations from embryo lysates

Translation activity of lysates prepared from post-fertilisation embryos was characterised. As already documented (Winkler *et al*., 1985; Colin *et al*., 1987; Hansen *et al*., 1987; Lopo *et al*., 1989), such lysates readily perform *in vitro* translation. In our experimental conditions, the kinetics of translation *in vitro* increased during the first 30 min (Fig. 2). After 60 min a plateau was reached. The neo-synthesised polypeptides ranged from 20 to > 150 kDa (Fig. 2, insert), reflecting translation of most, if not all, the endogenous mRNA species. Bands were clearly individualised indicating that mRNAs remain intact in the lysates, and are capable of directing the synthesis of complete proteins in the absence of premature termination.

The lysates were assayed for their ability to translate exogenous mRNA templates. When an exogenous mRNA, which encoded for the protein *Xenopus* EF-1 δ, was added to the lysates, no translation of EF-1 δ could be detected on autoradiography after electrophoresis of the total extract or of the immunoprecipitates performed with anti-EF-1δ antibody (Fig. 3 left, lanes A). In contrast, this mRNA was translated in the rabbit

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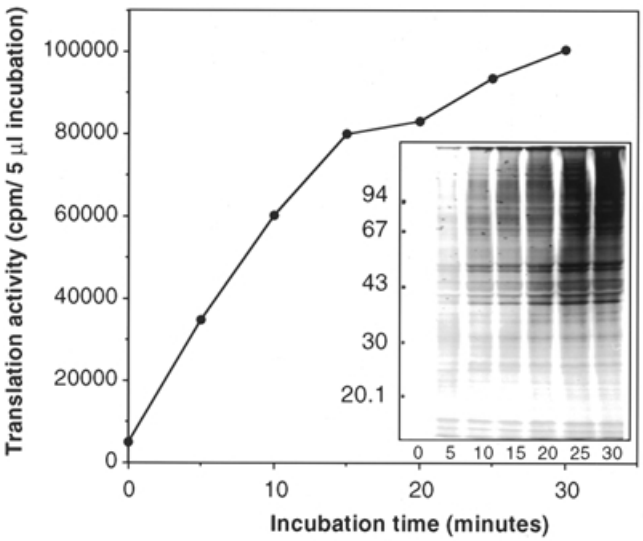

Figure 2 Translation activity in embryo lysate. Lysates were prepared from post-fertilisation embryos (90 min) as described in Materials and Methods. Incubations were performed in 200 µl. Translation activity was measured as [^{35}S]methionine incorporation into TCA-precipitable proteins on 5 µl aliquots taken every 5 min in duplicate, after addition of the lysate to the incubation mix. Inset: autoradiographs of [^{35}S]-labelled proteins resolved by SDS polyacrylamide gel electrophoresis determined from 10 µl aliquots of the same incubation. Positions of molecular weight markers are indicated in kDa.

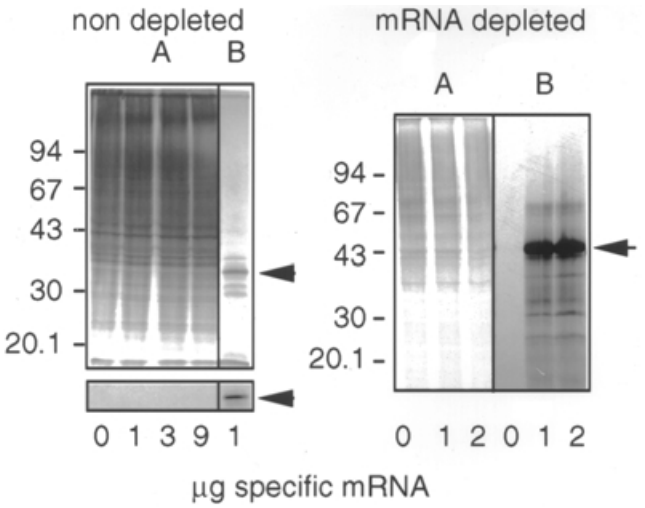

Figure 3 Protein synthesis from exogenous mRNAs. Specific mRNAs were added at the indicated amount to assays performed for 60 min with sea urchin lysates (lanes A) or with rabbit reticulocyte lysates as a control (lanes B). Upper left-hand panel shows autoradiography after SDS polyacrylamide gel electrophoresis of the incubations performed in the presence of endogenous mRNA encoding for the protein *Xenopus* EF-1 δ. The arrow points to the protein EF-1δ. The lower part shows an autoradiograph after SDS polyacrylamide gel electrophoresis of the proteins immunoprecipitated by an anti-*Xenopus* EF-1 δ specific antibody. Right-hand panel shows autoradiography of lysates (A, sea urchin; B, reticulocytes) depleted in endogenous mRNAs prior to the protein synthesis assay, performed in the presence of the indicated amount of specific mRNA encoding for the protein *Xenopus* EF-1 A. The arrow points to the protein EF-1 A. The positions of molecular weight markers are indicated in kDa.

reticulocyte lysate (Fig. 3 left, lane B). In the same way, the mRNA encoding for the protein EF-1 A, which was readily translated in rabbit reticulocyte lysate (Fig. 3 right, lanes B), was not detectable in the embryo lysates, even after depletion of the endogenous mRNAs by RNAse treatment (Fig. 3 right, lanes A). Therefore it appears that the lysates do not have the capacity to initiate translation of exogenous mRNAs, raising the question of the re-initiation of the endogenous mRNAs. The elongation inhibitors, puromycine and emetine, efficiently abolished protein synthesis (Fig. 4). In contrast, the initiation inhibitors 7-methylguanosine 5′-triphosphate and aurintricarboxylic acid had no effect on the rate of protein synthesis (Fig. 4).

Altogether, results show that the lysates perform protein elongation *in vitro* in the absence of re-initiation from mRNAs templates which have been initiated *in vivo*. The slope of the incubation kinetic curves reflects the elongation rate, whereas the level reached at the plateau reflects the amount of mRNAs which had previously been initiated *in vivo*.

Translation determinations after fertilisation

The overall increase in translation following fertilisation was determined from the comparison of translation activity of lysates prepared from unfertilised eggs and from post-fertilisation (90 min) embryos. The rate of protein synthesis from extracts of unfertilised embryos was 0.31% of total labelled methionine/hour/incubation (SD = 0.14; n = 19) compared with 5.8% of total labelled methionine/hour/incubation (SD = 4.7; n = 39) for the post-fertilisation embryo lysates, corresponding to an 18-fold increase.

Kinetics of *in vitro* translation were performed from lysates prepared every 15 min following fertilisation during the first two cleavages. The kinetics obtained for each post-fertilisation lysate were characterised by an increase and a plateau after 60 min. The curves could be separated into three groups, corresponding to increasing time post-fertilisation (Fig. 5). First, from fertilisation to 75 min (Fig. 5 left), an important increase in translation occurred with time, involving both translation initiation *in vivo* (plateau) and translation elongation rate (slope). The level of mRNA initiation *in vivo* was greatly increased as early as 15 min post-fertilisation and continued to increase until 60 min (around 6-fold). During the second interval period, from 90 to 180 min (Fig. 5 middle), translation

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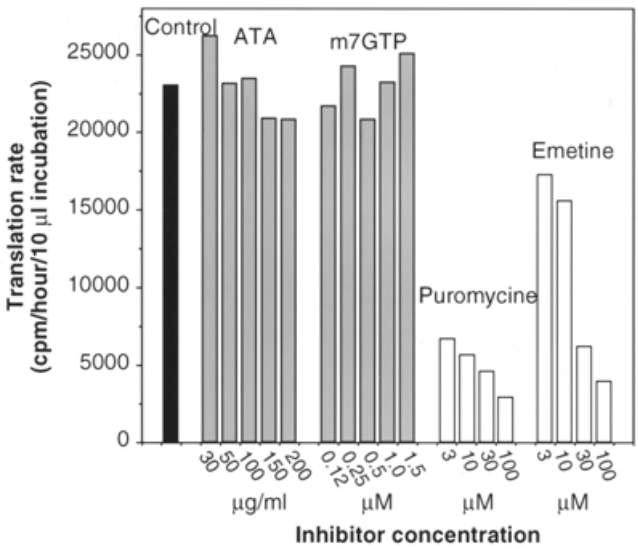

Figure 4 Effect of protein synthesis inhibitors on translation in the lysate. Translation activity was quantified by TCA precipitation after assays of the lysates in the absence (control, black) or presence of elongation (white) and initiation (grey) inhibitors at the indicated concentration. ATA, aurintricarboxylic acid; m7GTP, 7-methyl-guanosine 5′-triphosphate.

curves had comparable slopes and reached levels close to each other. The third period, from 195 to 240 min post-fertilisation, was again characterised by comparable kinetic curves, with higher slopes and higher plateau values than in the preceding period (Fig. 5 right). The second and third periods therefore revealed pauses in the rate of the translation increase, and are separated by a burst period. Regulation of the rate of translation elongation following fertilisation was characterised from the slopes of the kinetic curves (Fig. 6). The rate of elongation following fertilisation shows a 60 min burst (> 5-fold), a 75 min plateau or decrease, another burst (2-fold) occurring in a 60 min time period, and a further 45 min plateau. In five different experiments, the first pause in the elongation rate increase was observed during the activation phase of the histone H1 kinase when measured in parallel. The profiles of the major polypeptide synthesised *in vitro* determined at each time course interval of development were highly comparable, indicating that changes in translation involve all mRNA species (Fig. 6 insert).

Therefore, early development is characterised by an increase in translation associated with both initiation and elongation increases. The elongation increase is furthermore regulated by pauses in synchrony with the first cell divisions.

Effect of polyamines on translation

Optimal translation activity of the lysates was always obtained when extraction and dialysis buffer contained 1 mM spermidine, as already reported (Clemens, 1984), leading to 0.8 mM in the assay. Further addition of natural polyamines, spermine and spermidine, in the incubation medium lead to a dramatic inhibitory effect on translation. The effect was specific for spermine and spermidine (Fig. 7). Spermine (ID_{50} = 0.18; SD = 0.02; n = 4) was found to be more potent than spermidine (ID_{50} = 1.9; SD = 0.4; n = 7) at inhibiting translation. Analysis of the profile of the polypeptides synthesised in the presence of partial inhibitory concentrations of spermine and spermidine showed that inhibition affected all mRNA species to the same extent and therefore did not result in the disruption of polysomes (Fig. 8). Therefore, natural polyamines are strong potential regulators of elongation.

Polyamines could affect CK2 (Mamrack, 1989), a known effector of protein synthesis in other cells (Guerra & Issinger, 1999), which is present in the embryo lysate (Delalande *et al.*, 1999). The CK2 inhibitors heparin, DRB or 2,3-bisphosphoglycerate

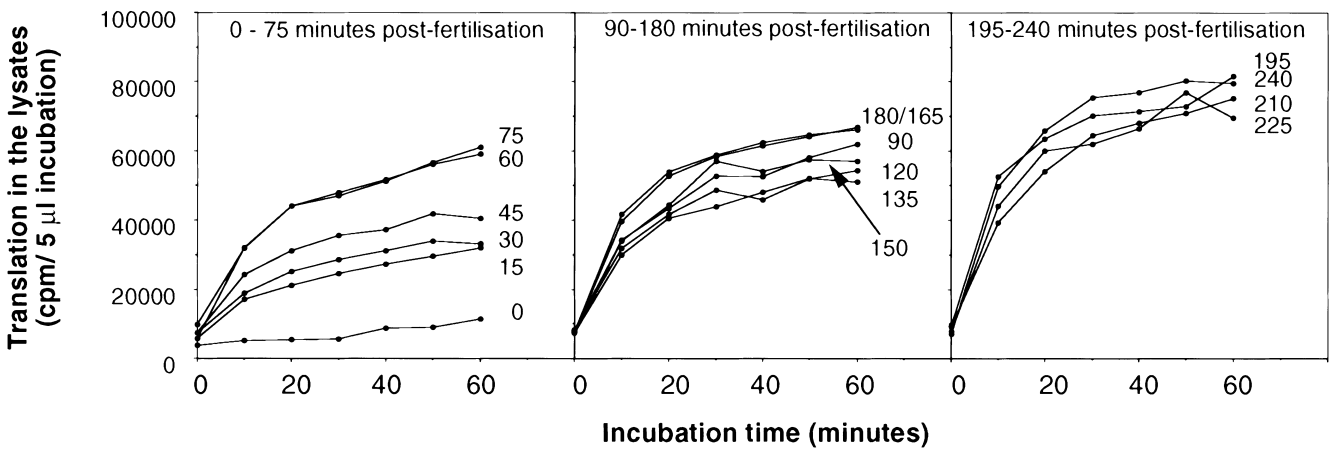

Figure 5 Kinetics of translation activity after fertilisation. Lysates were prepared every 15 min after fertilisation and were assayed for translation activity as in Fig. 2. The incorporation curves obtained were separated into three groups according to their kinetic characteristics.

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had no effect on translation activity of the lysates. Addition of excess CK2 to the lysates was ineffective in protein translation.

Polyamines therefore exert a dramatic effect on protein synthesis, which did not appear to be mediated by CK2, and which affects the elongation step of translation.

Discussion

Cell-free extracts, originally developed form reticulocyte lysates (Mathews, 1996), are currently used to dissect the mechanisms of the numerous translational events shown to occur during physiological processes such as development (Castagnetti *et al.*, 2000; Lie & MacDonald, 2000; Zamarro *et al.*, 2000). We have analysed changes in translation during the early development of sea urchin using a cell-free system. Our results indicate a large global increase in translation activity as already documented in other sea urchin lysates (Lopo *et al.*, 1988; Hille *et al.*, 1990; Jagus *et al.*, 1993). In our experiments, the overall increase was 18-fold, resulting in the progressive accumulation of neosynthesised proteins in the embryos (see Fig. 1). The properties of the lysates prepared in our experimental conditions allowed determination of elongation rates of the pre-initiated mRNAs *in vivo*. The relative increases in initiation and elongation were estimated to be 6-fold and 5-fold respectively in the first hour post-fertilisation. The originality of our results is evidence for characteristic elongation rate changes associated with the cell cycles of early development. The elongation rate increase was found to pause at the time of histone H1 kinase activation. Since histone H1 kinase corresponds to the M-phase CDK1/cyclin B (Borgne *et al.*, 1996), the pause is therefore concomitant with the entry into M-phase of the cell cycle. We conclude that, besides an increase in both initiation and elongation of translation following fertilisation, a cell-cycle-related regulation of elongation takes place.

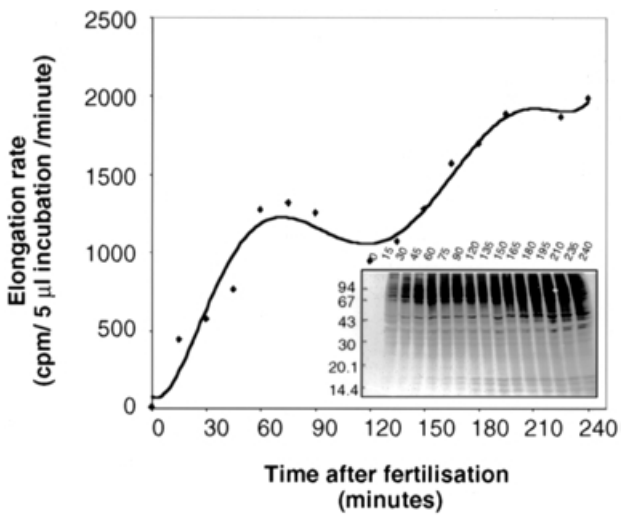

Figure 6 Elongation rates following fertilisation. Elongation rates were calculated from the 0 to 10 min slopes of the incorporation curves of Fig. 5, plotted as a function of time after fertilisation. Insert: autoradiograph of [^{35}S]-labelled proteins resolved by SDS polyacrylamide gel electrophoresis determined from 10 µl aliquots at the end of each incubation. Positions of molecular weight markers are indicated in kDa.

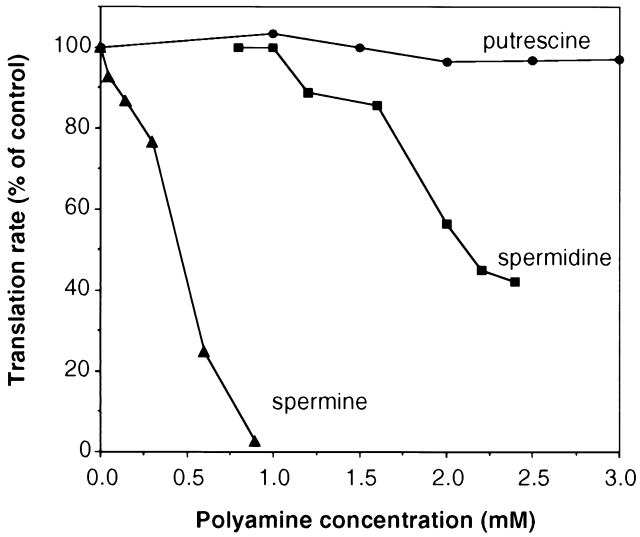

Figure 7 Polyamine effects on protein synthesis *in vitro*. Incorporation of [^{35}S]methionine was quantified by TCA precipitation from lysates prepared post-fertilisation (90 min) and assayed in standard conditions with the indicated concentration of the polyamines. All determinations were made in the presence of 0.8 mM spermidine due to the preparation protocol. In the spermidine experiment, the indicated polyamine concentration takes into account this basal amount.

We show that polyamines are very potent inhibitors of the elongation rate in the embryo lysate. Polyamines have been reported to affect translation in other cell-free extracts (Tabor & Tabor, 1984; Mezl *et al.*, 1986). The properties and specificity of the polyamine effects in the sea urchin embryo are comparable to those described in reticulocyte lysates (Mezl *et al.*, 1986) and were further shown to affect the elongation phase of translation from natural mRNA templates. The physiological targets of polyamines are still the subject of debate (Igarashi & Kashiwagi, 2000; Wallace, 2000). Recent compelling evidence indicates that polyamines are required for cell division, since their depletion leads to cell cycle arrest through expression of CDK inhibitors (Li *et al.*, 1999; Ray *et al.*, 1999; Alm *et al.*, 2000). In the same way, sea urchin embryos treated with an ornithine decarboxylase inhibitor, the enzyme responsible for polyamine biosynthesis, arrest their development at the first cell cycle (Kusunoki & Yasamasu, 1978). The level of

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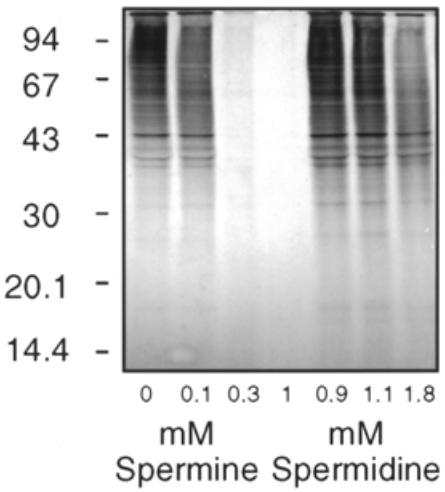

Figure 8 Profile of translation in the presence of polyamines. Autoradiographs of [^{35}S]-labelled proteins resolved by SDS polyacrylamide gel electrophoresis determined from 10 µl aliquots at the end of each incubation after incubation of the lysates in the presence of spermine or spermidine at the indicated concentrations. The positions of molecular weight markers are indicated in kDa.

polyamines was demonstrated to increase following fertilisation (Manen & Russell, 1973*a*; Kusunoki & Yasumasu, 1978). Furthermore, the polyamines spermine, spermidine and putrescine were demonstrated to undergo cycles in their concentration, being high in M-phase and dropping at cleavage (Manen & Russell, 1973*b*; Kusonoki & Yasamasu, 1976). We propose that natural polyamines could be implicated in the characteristic changes in translation that we demonstrated during early development. The increase in polyamines would be involved in the global increase in translation following fertilisation. At the higher level, corresponding to M-phase, polyamines would induce the pausing in the translation due to their inhibitory effect on elongation. The cyclic drop would then be necessary to relieve the pause. The physiological relevance for the cell cycle translation pausing and the molecular mechanism of the elongation transitory standby involving polyamines, remain to be determined.

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