Overexpression of S-adenosylmethionine decarboxylase (SAMDC) in early Xenopus embryos induces cell dissociation and inhibits transition from the blastula to gastrula stage

MIKIHIITO SHIBATA1, JUN SHINGA1, YUKUTO YASUHIKO1, MASATAKE KAI1, KIN-ICHIRO MIURA2, TOMOMI SHIMOGORI2, KEIKO KASHIWAGI3, KAZUEI IGARASHI3 and KOICHIRO SHIOKAWA1*

1Laboratory of Molecular Embryology, Department of Biological Sciences, Graduate School of Science, University of Tokyo, 2Institute for Biomolecular Science, Faculty of Science, Gakushuin University, Tokyo and 3Faculty of Pharmaceutical Sciences, Chiba University, Inage-ku, Japan

ABSTRACT Xenopus early embryos contain relatively low levels of S-adenosyl-methionine decarboxylase (SAMDC) and its mRNA. When SAMDC mRNA was injected into Xenopus embryos, it was preserved until the blastula stage and induced a large increase in SAMDC activity. The SAMDC-overexpressed embryos developed normally until the blastula stage but at the early gastrula stage cells which received the mRNA, dissociated autonomously and stopped synthesizing protein. In a hypotonic medium, the dissociated cells, and hence whole embryos, autolyzed. However, in isotonic media dissociated cells did not autolyze, although they did not divide and their DNA and RNA synthesis activity was greatly inhibited. The effects of SAMDC overexpression were abolished by coinjection of ethylglyoxal-bis(oxamidazole) (EBG), a specific inhibitor of SAMDC. In SAMDC-overexpressed embryos the level of putrescine decreased and that of spermidine increased, though to limited extents, resulting in a considerable decrease in the putrescine/spermidine ratio. However, direct injection of spermidine did not mimic the effect of SAMDC overexpression, and putrescine coinjected with SAMDC mRNA to maintain the normal putrescine/spermidine ratio did not rescue the embryos. Conversely, the level of S-adenosylmethionine (SAM) greatly decreased and coinjection of SAM, which restored the level of SAM, rescued the embryos. We concluded that in SAMDC-overexpressed embryos a SAM-deficient state was induced and this caused cell dissociation and inhibition of transition from the blastula to gastrula stage. We suggest that the SAM-deficient embryos obtained in the present study provide a unique system for studying the cellular control mechanism underlying the blastula-gastrula transition.

KEYWORDS: SAMDC overexpression, arrest at early gastrula, SAM, polyamines, cell dissociation, apoptosis

Introduction

SAMDC is one of the key enzymes in polyamine metabolism. It converts S-adenosylmethionine (SAM) into decarboxylated SAM (dcSAM), which serves as an aminopropyl donor to convert putrescine and spermidine into spermidine and spermine respectively (Tabor and Tabor, 1984; Pegg, 1986). In Xenopus embryos, SAMDC occurs at low levels (Shinga et al., 1996), whereas ornithine decarboxylase (ODC), another key enzyme producing putrescine from ornithine (Tabor and Tabor, 1984), occurs relatively abundantly (Osborne et al., 1989, 1991; Shinga et al., 1996). Both of these are the so-called high-turnover enzymes and their intracellular levels are regulated mainly at the transcriptional level (Tabor and Tabor, 1984; Pegg, 1986).

In Xenopus laevis, cDNA for ODC has been cloned and it has been shown that its mRNA occurs, like ODC, abundantly throughout embryogenesis (Osborne et al., 1991). cDNA for SAMDC, on the other hand, has not been cloned and little has been known about its mRNA expression in embryogenesis. We recently cloned cDNA for Xenopus SAMDC and showed that the mRNA for this enzyme occurs, like SAMDC, at low levels throughout early embryonic stages (Shinga et al., 1996).

Abbreviations used in this paper: SAMDC, S-adenosylmethionine decarboxylase; SAM, S-adenosylmethionine; dcSAM, decarboxylated SAM; ODC, ornithine decarboxylase; MBT, midblastula transition; EGB, ethylglyoxal-bis (guanylimidazole).

*Address for reprints: Laboratory of Molecular Embryology, Department of Biological Sciences, Graduate School of Science, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033, Japan. FAX: +81 3 3816 1965. e-mail: xishioka@biol.s.u-tokyo.ac.jp.
Fig. 1. Outer appearance of SAMDC mRNA-injected embryos.
Fertilized eggs were injected with 10 nl of either distilled water or SAMDC mRNA solution (1 ng/10 nl), and cultured in 0.1xSteinberg's solution, except in (F), (I), and (J), in which cultivation after the stage 6 was carried out in isotonic medium (1xSteinberg's solution or Sterans' complete medium). (A) Control and (B) SAMDC mRNA-injected embryos at the late blastula stage. (C) Control and (D) SAMDC mRNA-injected embryos at the early gastrula stage. In (D), an arrow head indicates the region in which dissociation occurred. (E) SAMDC mRNA-injected embryos autolysed at the late gastrula stage. (F) Dissociated cells of SAMDC mRNA injected-embryos cultured in Stearn's complete medium in a petri dish (vitelline membrane has been removed). Animal(G) and vegetal (H) views of an early gastrula injected with SAMDC mRNA into only one of the blastomeres at the 2-cell stage. (I), (J) SAMDC mRNA injected embryos cultured in 1xSteinberg's solution until the early gastrula (I) and the neurula stage (J). Arrows indicate that the region which underwent morphogenetic changes remained intact during the culture. Scales are not the same, but all control embryos were ca. 1.2 mm in diameter.
When we analyzed polyamine content in growing oocytes, oocytes during maturation and developing embryos of *Xenopus*, we found that, while the polyamine composition in oocytes is "eukaryote-type" (high putrescine and high spermine content), it changes during oocyte maturation to "E. coli-type" (high putrescine, but very low spermine content), and the E. coli-type polyamine composition is maintained throughout early embryogenesis (Osborne *et al.*, 1993; Shinga *et al.*, 1996). These results were compatible with the low levels of SAMDC and its mRNA and also with the high levels of ODC and its mRNA in *Xenopus* early embryos.

The change in the polyamine composition from the eukaryote-type to E. coli-type which occurred during oocyte maturation was quite remarkable, and therefore, we assumed that for *Xenopus* embryos to develop normally they must keep the E. coli-type polyamine composition. We expected that development of *Xenopus* embryos might be affected when their polyamine composition was shifted artificially from the E. coli-type to eukaryote- or oocyte-type.

One of the ways to induce such a change in polyamine composition in *Xenopus* embryos would be to overexpress SAMDC, since this enzyme is expected to accelerate the conversion of putrescine to spermidine and spermidine to spermine by increasing the level of dcSAM, the natural aminopropyl donor (Tabor and Tabor, 1984; Pegg, 1986). Thus, we performed experiments to microinject *in vitro* transcribed SAMDC mRNA into *Xenopus* fertilized eggs. However, our results showed that the microinjection of SAMDC mRNA does not alter the polyamine composition to the oocyte-type, although the microinjection results in an increase in the level of spermidine and a decrease in the level of putrescine.

Quite unexpectedly, however, we found that cells which receive SAMDC mRNA undergo dissociation autonomously at the early gastrula stage and SAMDC mRNA-injected embryos suddenly cease development, despite the fact that the mRNA-injected cells cleave and develop apparently normally until the late blastula stage. In the present paper, we describe this novel phenomenon observed with SAMDC-overexpressed *Xenopus* embryos, and present evidence which shows that the effects were induced specifically by the decrease in the level of SAM caused by the overexpression of SAMDC.

**Results**

**Effects of injection of SAMDC mRNA on early embryonic development**

We injected 1 ng of SAMDC mRNA into the animal side of *Xenopus* fertilized eggs, and cultured the injected embryos in 1xmodified Barth's solution (MBS) until stage 6, and thereafter in 0.1xSteinberg's solution. The mRNA-injected embryos cleaved and developed normally until the late blastula stage (Fig. 1A,B). At the early gastrula stage, however, animal side cells in the injected embryos separated from each other, became round and detached from the embryo (Fig. 1C,D). The size of some dissociated cells was apparently larger than those of normal-looking neighboring cells. The dissociated cells soon ruptured, and whole embryos underwent autolysis (Fig. 1E) before control embryos reached the late gastrula stage (not shown). In the embryos shown in Figure 1D, cells at the vegetal and marginal regions appeared normal and invagination took place as in the control (not shown), probably because the mRNA was not distributed in these regions.

**Fig. 2. Outer appearance of early gastrulae injected with SAMDC and β-galactosidase mRNA.** (A) Uninjected control embryos. (B) Embryos injected with 0.5 ng/egg of β-galactosidase mRNA alone. (C and D) Embryos injected with 0.5 ng/egg of SAMDC and 0.5 ng/egg of β-galactosidase mRNA together into either animal (C) or vegetal (D) side just after fertilization. Only dissociated cells were stained with X-gal. In (D) the larger arrow head indicates the vegetal pole region and the smaller arrow head indicates the animal pole region. All the embryos were cultured in 1xSteinberg's solution throughout stages.

When 1 ng of SAMDC mRNA was injected into only one of the blastomeres at the 2-cell stage, both uninjected and injected blastomeres cleaved and reached the blastula stage apparently normally (not shown). At the early gastrula stage, cell dissociation.
Fig. 3. Sections of SAMDC mRNA-injected embryos at different stages. One ng of SAMDC mRNA was injected into fertilized eggs at the animal side and cultured in 0.1x Steinberg’s solution after stage 6. Uninjected (A, C, and E), and SAMDC mRNA-injected (B, D, F and H) embryos at early blastula (A and B), late blastula (C and D), early gastrula (E and F) and late gastrula (H) stages are shown. In (G), an embryo was injected with SAMDC mRNA into only one of the animal side blastomeres at 8-cell stage and sectioned at the very early gastrula (pigment gastrula) stage. (H), An embryo at early gastrula stage, in which completely dissociated cells in the perivitelline space appear to be already in the process of autolysis.

agains took place, but only in a half portion of the injected embryo, leaving the other half of normal appearance (Fig. 1G). Invagination did not take place in the dissociated half (Fig. 1H, left half portion), while the other normal-looking half of the embryo underwent invagination (Fig. 1H, right half portion). The effect of SAMDC mRNA stronger in Figure 1G,H than in 1D may be due to the wider distribution of the injected mRNA within the blastomere. The half-dissociated embryos (Fig. 1G,H) soon underwent autolysis as in Figure 1E. However, when the vitelline membrane was removed and the undissociated half of the embryo was cultured in 0.1x Steinberg’s solution, it underwent a kind of wound healing and continued its subsequent, though not normal, development (not shown). Therefore, we assume that the dissolution of the normal-looking part of the SAMDC mRNA-injected embryo within the vitelline membrane (Fig. 1E) must be due to the toxic effects of substances liberated from the dissociated and ruptured cells.

We repeated the experiment to inject 1 ng of SAMDC mRNA into an uncleaved fertilized egg and cultured the embryos in an isotonic medium (either 1x Steinberg’s solution (Fig. 1I,J) or Stearns’ complete medium (not shown)) throughout stages. Again, cell dissociation occurred when control embryos reached the early gastrula stage (Fig. 1I), suggesting that cell dissociation is autonomous and not induced by the combined effects of SAMDC mRNA-injection and cultivation in the hypotonic medium (0.1x Steinberg’s solution). In this case (cultivation in the isotonic medium throughout stages), however, dissociated cells did not autolyse and remained intact at least in their outer appearance, for over 12 h. Furthermore, the remaining normal-looking cells underwent morphogenesis, although it was not normal (Fig. 1J, arrows).

When the vitelline membrane was removed from these embryos at the early gastrula stage, and the dissociated cells were cultured in Stearns’ complete medium, each of the dissociated cells maintained its spherical appearance for at least 12 h (Fig. 1F). During this culture, however, neither reaggregation nor cell division took place (the number, size and spatial arrangement of dispersed cells did not change throughout the culture) (not shown), although cells dissociated from control embryos using Stearns’ dissociating medium divided extensively and formed aggregates of various sizes (not shown, but see Shiokawa et al., 1985a). These dissociated cells appeared to maintain the integrity of their cell membrane, since they did not take up methylene blue, unlike those treated once with 90% ethanol (data not shown), and never released yolk granules or other cytoplasmic substances throughout the culture of 12 h (cf. Fig. 1F). Based on these observations, we concluded that the immediate rupture of dissociated cells followed by dissolution of the whole embryo observed in 0.1x Steinberg’s solution is due to osmotic shock caused by the hypotonic medium.

To discriminate cells derived from the mRNA-injected and uninjected regions of the egg cytoplasm, mRNAs for SAMDC and bacterial β-galactosidase were mixed and injected into either the animal or vegetal side of fertilized eggs (at 0.5 ng/egg). When embryos injected with β-galactosidase mRNA alone were examined at the early gastrula stage, only a part of the embryo expressed the blue color (Fig. 2A,B), indicating that the injected mRNA was not evenly distributed within the embryo (as expected from the data in Fig. 1). Similar results were obtained with embryos co-injected with SAMDC and β-galactosidase mRNAs, irrespective of the injected region (animal side injection (Fig. 2C) and vegetal side injection (Fig. 2D)). Interestingly, the dissociated cells were those colored blue and the undissociated normal-looking cells were not colored, suggesting that SAMDC mRNA was not distrib-

| TABLE 1 | MACROMOLECULAR SYNTHESIS IN DISSOCIATED CELLS FROM SAMDC mRNA-INJECTED EMBRYOS AT EARLY GASTRULA STAGE |
|----------------|---------------------------------|---------------------------------|
| Incorporation of radio label into acid-insoluble fraction (cpm/mg protein) | Radioactive precursors | Uninjected embryos | SAMDC mRNA-injected embryos |
| 3H-Thymidine | 1440±30 | 715±79 |
| 3H-Uridine | 502±17 | 264±25 |
| 14C-Leucine | 493±295 | 905±28 |

Embryos were injected with 0.5 ng of SAMDC mRNA at the 2-cell stage into both of the blastomeres. Four independent experiments were carried out and mean values and standard deviation are shown. Protein amount was determined according to the method by Lowry et al. (1951)
Effects of SAMDC overexpression in Xenopus embryos

Fig. 4. Dosage effect of SAMDC mRNA on development. Fertilized eggs were injected with 0 (injected with only 10 nl of distilled water), 0.01, 0.1, 1.0, or 10 ng/egg of SAMDC mRNA and cultured in 0.1xSteinberg's solution after stage 6. The number of embryos used was 100, 92, 50, 71, and 118, respectively. As a control experiment, 199 embryos were injected with 1 ng/egg of mRNA for type IIA activin receptor (ActR). The percentage of normal-looking embryos is shown at each time point.

Histological examination of SAMDC mRNA-injected embryos

We injected SAMDC mRNA (1 ng) into fertilized eggs at the animal side and embryos were histologically examined at early (Fig. 3A,B) and late (Fig. 3C,D) blastula stages. No difference was observed in the size and arrangement of constituent cells and the appearance of the blastocoel between the control and SAMDC mRNA-injected embryos. When we examined embryos at the early gastrula stage, a large number of dissociated cells were found in the blastocoel (Fig. 3E,F). The dissociated cells were round (Fig. 3F) in shape, like those at the surface of the embryo (see Figs. 1D,G and 2C,D). In the embryo shown in Figure 3F, the injected SAMDC mRNA was probably not distributed into marginal and vegetal regions, and invagination took place to the extent almost comparable to that in the control embryos (Fig. 3E).

Figure 3G shows the blastocoel of an embryo at the pigment gastrula stage which had been injected with 1 ng of SAMDC mRNA into only one of the animal side blastomere at the 8-cell stage. In this embryo, only cells at the right half portion of the animal side separated from each other, but unlike those in Figure 3F, they were not round in shape. Probably, these cells were at the initial step of dissociation, since the outermost cells were still connected by surface coat, which had not been disrupted yet. Therefore, we concluded that in SAMDC mRNA-injected embryos cell disaggregation starts at the pigment gastrula stage from the inside of the embryo, before it becomes apparent from the outside.

By contrast, in the embryo examined at the early gastrula stage (Fig. 3H), the surface coat was not observed and cell layers in the animal side were almost completely disrupted. The round cells observed here were extremely large, probably due to swelling of cells in the process of autolysis caused by the hypotonicity of the medium (0.1xSteinberg's solution). Thus, the blastocoel was filled with dispersed yolk granules and cell debris derived from autolyzed cells. We assume that this embryo was at the initial step of the whole embryo dissolution.

Effects of different doses of SAMDC mRNA and other mRNAs

We injected different amounts of SAMDC mRNA into fertilized eggs and examined embryos cultured in 0.1xSteinberg's solution after stage 6. SAMDC mRNA did not interfere with the development at 0.01 ng/egg (Fig. 4). When the dose was increased from 0.1 to 10 ng/egg, the relative number of abnormal embryos increased roughly in a dosage-dependent manner. However, in the wide range of the dosage tested (100-fold), cell dissociation took place constantly at the gastrula stage. This result suggests the...
occurrence of a SAMDC mRNA-sensitive step during the transition from the blastula to gastrula stage.

Injection of 1 ng/egg of mRNA for Xenopus type II A activin receptor did not inhibit development (Fig. 4), although tadpoles obtained here occasionally had a secondary axis due to the ectopic expression of activin receptor (cf. Kondo et al., 1991). We also tested two other Xenopus mRNAs (mRNAs for nrp-1 (Richter et al., 1990) and eIF4E (Wakiyama et al., 1995)) at doses of 0.5 and 1 ng/egg, but again these had no effect on development (data not shown). We also tested the effect of the injection of SAMDC mRNA (1 ng/egg) without a cap structure (mRNA transcribed in the absence of a cap analog), but this was also not effective (data not shown). Therefore, we concluded that the cell autonomous disassociation observed was induced by the specific function of the injected SAMDC mRNA.

**Macromolecular synthesis in cells dissociated by SAMDC mRNA injection**

We injected 0.5 ng of SAMDC mRNA into both of the blastomeres at the 2-cell stage and cultured the injected embryos in 1xSteinberg's solution after stage 6. Approximately 80% of the constituent cells were dissociated at late gastrula stage. We eliminated the vitelline membrane and used the cells from the whole embryos (most of them were dissociated autonomously as above) for the experiment to determine if the SAMDC mRNA-injected embryos retain the activity to synthesize DNA, RNA and protein by culturing them in Stearns' complete medium (cf. Fig. 1F). Control cells here were obtained by dissociating normal embryos with the Stearns' dissociating medium (Shiokawa and Yamana, 1967).

When these cells were labeled for 3 h with 3H-thymidine, 3H-uridine or 14C-leucine, it was found that cells from SAMDC mRNA-injected embryos incorporated 3H-uridine and 3H-thymidine into their acid-insoluble fraction at about the 50% level of the control, whereas incorporation of 14C-leucine was reduced by at least 80% (Table 1). In the labeling experiment with 3H-thymidine, RNAs were extracted and fractionated on agarose-polyacrylamide gels, and it was confirmed that the label was distributed in all the major RNA species (rRNA, tRNA, and heterogeneous mRNA-like RNA) (data not shown). Since protein synthesis appeared to be most severely inhibited, we analyzed the incorporation of 14C-leucine also in embryos at earlier stages, when SAMDC mRNA-induced cell dissociation did not take place. For this experiment, we injected 0.5 ng/egg of SAMDC mRNA into both of the blastomeres at the 2-cell stage, and after dissociating control and SAMDC mRNA-injected embryos at stages ranging from early blastula to gastrula stage, labeled them with 14C-leucine in the Stearns' complete medium for 2 h. As shown in Table 2, protein synthesis in SAMDC mRNA-injected embryos started to be inhibited from the mid blastula stage (stage 8). Therefore, we concluded that the inhibition of protein synthesis precedes the initiation of cell dissociation.

**Changes in the levels of SAMDC mRNA and SAMDC in injected embryos**

We injected 0.5 ng of SAMDC mRNA into fertilized eggs, and carried out Northern blot analysis using the RNA extracted from embryos at 2 h after injection (the 8-cell stage). The amount estimated for the recovered mRNA was 0.4 ng/embryo, which was in close agreement with the amount injected (0.5 ng) (for the

| TABLE 2 | PROTEIN SYNTHESIS IN SAMDC mRNA-INJECTED EMBRYOS AT VARIOUS STAGES |
|----------------|--------------------|----------------|
| **Nieuwkoop-Faber stages** | **Uninjected embryos** | **SAMDC mRNA-injected embryos** |
| Stage 7 | 287±4 | 263±24 |
| Stage 8 | 456±24 | 305±8 |
| Stage 9 | 401±27 | 260±14 |
| Stage 10 | 405±41 | 188±2 |

Embryos were injected with 0.5 ng of SAMDC mRNA at the 2-cell stage into both of the blastomeres. Four independent experiments were carried out and mean values and standard deviation are shown. Protein amount was determined according to the method by Lowry et al. (1951).
Effects of SAMDC overexpression in Xenopus embryos

Fig. 7. Changes in the level of three polyamines in control and SAMDC mRNA-injected embryos. Fertilized eggs were injected with 0.5 ng/egg of SAMDC mRNA into both of the blastomeres at the 2-cell stage and contents of putrescine, spermidine, and spermine were determined in the control and SAMDC mRNA-injected embryos at different stages. Two independent experiments were carried out and mean values and standard deviation are shown.

TABLE 3

<table>
<thead>
<tr>
<th>Dosage of SAMDC mRNA (ng/egg)</th>
<th>Hours after injection</th>
<th>Developmental stage (Nieuwkoop-Faber stage)</th>
<th>SAMDC activity (pmoles 14CO2/1h/embryo)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Uninjected embryos</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>4-cell stage (3)</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Blastula (8)</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>Gastrula (10.5)</td>
<td>0.78</td>
</tr>
<tr>
<td>0.1</td>
<td>3</td>
<td>Morula (6)</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Blastula (9)</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>Gastrula (11)</td>
<td>0.60</td>
</tr>
</tbody>
</table>

SAMDC mRNA was injected into fertilized eggs at the indicated amount just after fertilization, and the activity of SAMDC was determined using 100 embryos at the indicated stage.
mRNA (data omitted). These results indicate that cell dissociation observed in SAMDC mRNA-injected embryos was due to the overexpression of SAMDC.

Changes in polyamine levels in SAMDC mRNA-injected embryos and failure of putrescine to rescue the SAMDC-overexpressed embryos

We injected 0.5 ng of SAMDC mRNA into both blastomeres of 2-cell stage embryos and followed the changes in the levels of polyamines in embryos cultured in 1x Steinberg's solution throughout the stages. In control embryos, the levels of putrescine, spermidine, and spermine (Fig. 7) were consistent with previous data (Shinga et al., 1996). In embryos injected with SAMDC mRNA, the level of putrescine became lower and that of spermidine became higher than the control value at the gastrula stage and the decrease in the level of putrescine was ca. 25% (by 400 pmoles/embryo) whereas the increase in the level of spermidine was ca. 25% (by 200 pmoles) of the respective control value. Similar results were obtained by culturing embryos in 0.1x Steinberg's solution after stage 6 (not shown), suggesting that cells dissociated in embryos cultured in a hypotonic medium do not change their polyamine composition prior to rupture by osmotic shock. Thus, injection of SAMDC mRNA induced a significant, but limited extent, of conversion of putrescine into spermidine and there was no evidence for the conversion of polyamine composition from E. coli-type to eukaryote- or oocyte-type, despite of our initial expectations.

Figure 7 clearly shows that, as a result of the change in the levels of putrescine and spermidine, the intra-embryo putrescine/spermidine ratio, which was usually ca. 2.0, was lowered to 1.0 by the time of cell dissociation. Therefore, it was suspected that this change in the ratio might be the cause of the cell dissociation, since Osborne et al. (1993) reported the importance of the maintenance of the normal putrescine/spermidine ratio in Xenopus early embryos. Thus, we carried out experiments to inject into fertilized eggs 1 ng/egg of SAMDC mRNA together with 1 nmole of putrescine (roughly equivalent to twice as much spermidine expected to be increased in the SAMDC mRNA-injected embryo) (cf. Fig. 7). We confirmed that injection of 1 nmole of putrescine alone does not inhibit development (data not shown), and also that it increases the intra-embryo level of putrescine (by ca. 50%) to restore the normal putrescine/spermidine ratio (ca. 2) within the SAMDC mRNA-injected embryos (Fig. 8). However, in the embryos co-injected with SAMDC mRNA and putrescine, cell dissociation took place and development was arrested at the early gastrula stage just as in embryos injected with SAMDC mRNA alone (Fig. 6D-F). Therefore, the decrease in the putrescine/spermidine ratio in SAMDC mRNA-injected embryos may not be the cause of the cell dissociation.

Rescue of SAMDC mRNA-injected embryos by the injection of SAM

We injected 1 ng of SAMDC mRNA into fertilized eggs and examined the level of SAM at the late blastula stage. HPLC profiles of the acid-soluble fraction (not shown) demonstrated that the level of SAM was reduced by ca. 80% in SAMDC mRNA-injected embryos. The level of dcSAM did not exceed background levels in both control and SAMDC mRNA injected-embryos (not shown).

Since a substantial decrease was observed in the level of SAM, we tested if this decrease in the level of SAM might be the cause of cell dissociation (and developmental arrest). For this purpose, we injected 200 pmoles of SAM together with SAMDC mRNA into fertilized eggs, and followed the development of the injected embryos by culturing them in 0.1x Steinberg's solution after stage 6. Initially, we performed this experiment using 1 ng/egg of SAMDC mRNA, but the co-injected SAM did not rescue cell dissociation (data not shown, but see Fig. 1D), probably because of the rapid conversion of the injected SAM into dcSAM.

| TABLE 4 | LEVEL OF SAM IN EMBRYOS INJECTED WITH SAMDC MRNA ALONE OR SAMDC MRNA AND SAM |
|---------|-------------------------------|-----------------|-----------------|
|         | content of SAM (pmoles/embryo) | SAMDC mRNA     | SAMDC mRNA     |
|         |                               | injected       | and SAM-injected |
| Control |                               |                 |                 |
| Experiment 1 | 124                   | 48              | 285             |
| Experiment 2 | 87                      | 41              | 278             |

SAMDC mRNA (0.1 ng/embryo) was injected alone or together with SAM (200 pmoles/embryo) into fertilized eggs, and the content of SAM was determined using 50 embryos per sample at late blastula.
We performed the same experiment using 0.1 ng/egg of SAMDC mRNA. This amount of SAMDC mRNA was 10-fold lower than that used initially, but was expected to induce cell dissociation in at least 50% of the injected embryos according to the results in Figure 3. The data obtained here clearly showed that exogenously-supplied SAM completely suppresses cell dissociation (Fig. 6G-I), permitting all the SAMDC mRNA-injected embryos to develop into normal tadpoles (Fig. 9). The lower dose of SAMDC mRNA (0.1 ng/egg) alone reduced the level of SAM by 50-60%, whereas coinjection with SAM increased the level of SAM within the embryo to the double of the controls (Table 4). In addition the level of SAMDC in the embryos coinjected with SAMDC mRNA (0.1 ng/egg) and SAM (200 pmole/egg) was as high as that in the embryos injected with SAMDC mRNA (0.1 ng/egg) alone (data not shown, but see Table 3). Thus, we reached the conclusion that the injection of SAMDC mRNA induces deficiency of SAM, and this, primarily, is the cause for the cell dissociation followed by cessation of normal development.

Injection of dcSAM (200 pmole/egg) into fertilized eggs had no effect on the development of embryos, despite an increase in the level of dcSAM from undetectable to ca. 80 pmole/egg. Therefore, we assumed that dcSAM, which could be increased at least transiently by SAMDC overexpression, may not be toxic by itself to development, and may not be the cause for the cell dissociation.

Discussion

In the experiments presented in this paper, we overexpressed SAMDC in Xenopus embryos by microinjection of in vitro transcribed Xenopus SAMDC mRNA and examined the development of the mRNA-injected embryos. We demonstrated that cells overexpressed with SAMDC started to dissociate autonomously from the pigment gastrula stage from the inside of the embryo and, as a result, development was arrested or severely damaged during the gastrula stage. When we performed these experiments under the conditions routinely used for experiments of this type (i.e., 0.1xSteinberg's solution), dissociated cells and embryos underwent autolysis soon after the appearance of dissociated cells. Initially, we thought that the two processes, namely cell dissociation and autolysis of the whole embryos, were tightly linked, and therefore inseparable. However, as clearly shown in the experiments using isotonic media (1xSteinberg's solution and Stearns' complete medium), this was not the case and cell autolysis was simply due to osmotic shock caused by the hypotonicity of the medium. Dissociated cells in which SAMDC was overexpressed, were maintained apparently intact for more than 12 h preserving for at least 3 h low levels of RNA synthesis activity and ability to incorporate thymidine, although their protein synthesis was almost totally inhibited. Thus, dissociated cells are viable at least until the moment when they dissociate. In other words, we assume that cell dissociation was not the result of simple cell death brought about by SAMDC overexpression.

The overexpression of SAMDC was detected from 1 h after its mRNA injection, a finding which is compatible with the well known activation of maternal protein-synthetic-machinery at oocyte maturation (Ecker and Smith, 1968; Woodland, 1974). Therefore, the effect of SAMDC overexpression could begin shortly after the injection of its mRNA. However, cell dissociation occurred consistently at the early gastrula stage, despite the dose-dependent response of cell dissociation, developmental arrest and the extent of SAMDC overexpression. Taken together, it is tempting to consider that there is some "time-switched" mechanism which triggers the cell dissociation at the end of the blastula stage, or shortly after midblastula transition (MBT) (Newport and Kirschner, 1982), which involves developmentally important cellular events such as the change in cell cycle, in cell motility, and total transcriptional activity (on a per-embryo basis, at least (Shiokawa et al., 1994)).

The cell dissociation (and developmental arrest) induced by SAMDC overexpression is a quite unique phenomenon. It is different from the developmental arrest reported to be induced by injection of metabolic inhibitors, such as actinomycin D, cordycepin, and trichostatin A since the developmental stage of embryos at which the effects of these inhibitors become apparent varies with dosage (Shiokawa et al., 1983; Almouzni et al., 1994). Also, the arrest induced by SAMDC overexpression is different from that induced by the treatment of embryos with cytotoxic agents, such as colchicine, cytochalasin B, and podophyllotoxin since all of these stop cell division during the cleavage stage at high doses (Shiokawa et al., 1985b; Takeichi et al., 1985). Furthermore, the arrest induced by SAMDC overexpression is different from that found during the gastrula arrest in maternal-defect mutant embryos (Ikenishi and Tsuzaki, 1988; Shiokawa et al., 1988), since, unlike the mutant cells, SAMDC-overexpressed blastula cells did not form heavily vacuolated nuclei (Ikenishi and Tsuzaki, 1988).

At the beginning of this study, we expected that SAMDC overexpressed within the embryo would greatly accelerate the conversion of putrescine into spermidine and spermine into spermine, thereby changing the polyamine composition from the E. coli-type to euakaryote- or oocyte-type. However, although a
change in the polyamine composition was observed, this was only to a limited extent. Probably, there are some other factors necessary to be altered in order to shift the polyamine composition from the E. coli-type to eukaryote- or oocyte-type. The most probable candidates are the levels of spermine and spermidine synthetases (Tabor and Tabor, 1984; Pegg, 1986), a possibility which is now under investigation.

Concerning the possibility that the alteration in the polyamine composition might be the main cause of the observed cell dissociation and developmental effects, Osborne et al. (1993) reported interesting findings. According to these authors, the injection of 250 pmol of spermidine (or spermine) into Xenopus fertilized eggs induces highly toxic effects on the injected blastomeres and stops cleavage at 2 or 4-cell stage to kill embryos immediately. However, these authors also reported that the injection of spermidine (or spermine) at lower doses has no effect on development. We repeated these experiments (K. Kondo and K. Shiokawa, unpublished observations), and obtained similar results to Osborne et al. (1993) (From our results, at least 300 pmol, but not lower doses, of spermidine (or spermine) exerted the embryo-killing effects). Thus, apparently, there is a large difference in the effect on development of the increased level of spermidine within the embryo between the SAMDC mRNA-injecting experiment and directly polyamine-injecting experiment: Cells directly injected with an effective amount of spermidine stopped development and immediately died (actually completely dissolved within the vitelline membrane in 1xSteinberg’s solution), but those injected with SAMDC mRNA and induced to have the increased level of spermidine stopped cell division but remained apparently intact for at least 12 h after the dissociation in the same 1xSteinberg’s solution. Therefore, the cell-dissociating effect observed with SAMDC mRNA injection may not be attributed to the increase in the level of the spermidine.

In their microinjection experiments with Xenopus fertilized eggs, Osborne et al. (1993) also showed that the toxic effect of exogenously-injected spermidine (250 pmol/egg) was completely abolished when a twice as much amount of putrescine (500 pmol) was injected together with spermidine so that the injection did not alter the normal putrescine/spermidine ratio (ca. 2) within the embryo. Since there was a large decrease in the putrescine/spermidine ratio in the SAMDC mRNA-injected embryos (Fig. 7), we assumed that the decrease in the ratio might be the cause of the cell dissociation. However, exogenously-supplied putrescine did not rescue the embryos, although it restored the normal putrescine/spermidine ratio within the embryos. Therefore, we concluded that neither the increase in the level of spermidine nor the decrease in the putrescine/spermidine ratio could explain the effect of the overexpression of SAMDC.

Subsequently, we determined the level of SAM, the substrate of SAMDC, and found that it was greatly reduced in SAMDC-overexpressed embryos. Furthermore, we performed experiments to coinject SAM and SAMDC mRNA and found that coinjection of SAM completely rescued the development of SAMDC-overexpressed embryos. Therefore, we concluded that it is the deficiency of SAM that induces cell dissociation. We assume that inhibition of methylation is probably involved, a possibility which is now under investigation.

As for the mechanism of the induction of cell dissociation, inhibition of cadherin synthesis might be involved. In particular, for Xenopus embryos to maintain normal cell adhesion and development it is necessary to maintain normal protein synthesis activity (Shiokawa et al., 1983), and the requirement for a supply of maternally-synthesized cadherins for maintenance of normal cellular arrangement and subsequent formative movement in blastulae has been shown in the case of EP-cadherin (Heasman et al., 1994) and XmN-cadherin (Hojyo et al., 1998). However, it is also well known that detachment from neighboring cells is the initial behavior of cells entering the apoptotic process. In this relation, it was observed that, while dissociated cells in embryos cultured in a hypotonic medium ruptured and scatter their cellular contents within the vitelline membrane to induce total embryo death, those cultured in the isotonic medium do not undergo such a "dirty" cell death, but merely cease cell division and remain inert, not scattering their cytoplasmic substances to the outside of cells. Furthermore, in preliminary experiments, we observed abnormal condensation of chromatin in some of the nuclei of SAMDC mRNA-affected cells (M. Shibata, H. Fukamachi and K. Shiokawa, unpublished observations). Thus, it is also plausible that the induction of cell dissociation observed here simply reflects the beginning of apoptotic cell death, induced under the conditions of SAM deficiency, rather than the specific depletion of cadherins from the cell surface. We observed that the inhibition of protein synthesis precedes the initiation of cell dissociation, and this inhibition in protein synthesis becomes maximum after cell dissociation, which probably secondarily induces the inhibition of RNA and DNA synthesis. Therefore, we assume that for some unknown reasons the deficiency in SAM induces the inhibition of protein synthesis, and this probably switches on the program of apoptotic cell death. Thus, the dissociated cells are those destined to die, although the cells were not dead at the time of dissociation.

As stated above, overexpression of SAMDC induced cell dissociation consistently during the period of the blastula to gastrula stage. Thus, from the present results, it is apparent that the levels of both SAMDC and its mRNA in early embryos have to be kept low so as not to induce SAM deficiency, and allow the normal transition from the blastula to gastrula stage to occur. We expect that further analysis of the SAMDC-overexpressed embryos will provide us with important and unique information on the function played by SAM on the one hand, and on the yet unknown mechanism underlying the transition from the blastula to gastrula stage, on the other. With this in mind, important papers by Sible et al. (1997) and Hensey and Gautier (1997) appeared while our paper was in preparation. These authors described that apoptotic cell dissociation could be induced by the injection of cycloheximide and α-amanitin. It appears at present that, in the cases of Sible et al. (1997) and Hensey and Gautier (1997), the injection of metabolic inhibitors prevents the appearance of a survival factor(s), whereas in our case the SAM deficient state results in the appearance of a death factor(s) which probably directs cells to enter the apoptotic process.

Materials and Methods

In vitro transcription of mRNAs

The plasmid carrying the Xenopus SAMDC mRNA (XSD3) (pBluescript SK(-)-XSD3) (Shinga et al., 1996) was purified following the methods described by Sambrook et al. (1989). To obtain XSD3 ORF (open reading frame), we performed PCR using two oligonucleotides, SD-N (5'-ACCATGGAGATGGAGGAGAGCG-3') and SD-X (5'-GTCAGGATCACTCTGCTGTCG-3') as primers and Xenopus SAMDC cDNA (XSD3) as a template (Shinga et al., 1996). The ORF was subcloned into the Ncol and
XbaI sites in pSP36T (a gift from Drs. Amaya and Kirschner), a derivative of pSP64T (Krieg and Melton, 1984) that carried 5’- and 3’-UTRs (untranslated regions) of Xenopus β-globin mRNA. The construct obtained was linearized at the PvuII site and transcribed in vitro with SP6 RNA polymerase.

As reference mRNAs, Xenopus eukaryotic initiation factor (eIF4E) (in pSP36T) (Wakiyama et al., 1995) and Xenopus RNA-binding protein rp1-1 (in pSP36T) (Richter et al., 1990) were also prepared and transcribed essentially following the methods as above. Also, linearized plasmids carrying the bacterial β-galactosidase gene (in pGEM-7Zf(-)-LacZ) and XbaI type IIA activin receptor (XAR7) (in pBluescript II SK(-)-XAR7) (Kondo et al., 1991) were transcribed with T3 RNA polymerase (cf. Kondo et al., 1991).

Except in the experiment which tested the effect of the presence of the CAP structure in mRNA, all the mRNAs used were transcribed in the presence of a cap analog (7mG(5')ppp(5')G) (New England Biolabs). The sizes of the in vitro transcribed RNAs were confirmed by gel electrophoresis (data not shown). RNAs were dissolved in distilled water, and kept at -80°C until used.

**Embryos and mRNA microinjection**

Unfertilized eggs of Xenopus laevis were manually ovulated from gravid females, injected with a hyman chronic gonadotropin hormone, Gonatoropin (Teikoku Zoki). Eggs were artificially fertilized, and dejellied in 2% cysteine-HCl (pH 8.0) (see Koga et al., 1995). Eggs were kept in 0.1x Steinberg’s solution until used for microinjection (Shinga et al., 1996).

mRNAs were microinjected at 0.01 to 10 ng/egg in 10 nl of distilled water into the region specified in each experiment in 1x modified Barth’s solution (MBS), which contained 5% Ficol 400, 50 μg/ml streptomycin and 50 units/ml penicillin (Nakamura et al., 1996). Injected embryos were kept in 1xMBS until stage 6 (Nieuwkoop and Faber, 1967).

Injected embryos were transferred into 0.1x Steinberg’s solution at stage 6 for further culture. This was because cultivation of whole embryos in the isotonic medium quite often induces abnormality in embryos (e.g. shortened body length, reduction in size of head or tail, hyperformation of head part, etc) (Shiokawa, unpublished observations). However, in the experiments to prevent rupture of dissociated cells in embryos injected with SAMDC mRNA, 1x Steinberg’s solution was used in place of 0.1x Steinberg’s solution even after stage 6. The choice of the culture medium is specified in the text and/or figure legend for each experiment. All the embryos were kept in an incubator at 21°C.

**Light microscopic observation**

Embryos were fixed for 12 h with 10% neutralized formalin dissolved in 0.1x Steinberg’s solution, dehydrated in a graded series of ethanol, and embedded in paraffin. Samples were sectioned serially at 5 μm, stained with hematoxylin and eosin, and examined under a light microscope (Sameshima et al., 1968).

**Detection of β-galactosidase activity**

Embryos injected with mRNAs for bacterial β-galactosidase were fixed for 1 h in phosphate-buffered saline containing 2% formamide, 0.2% glutaraldehyde, 0.02% Triton X-100 and 0.01% sodium deoxycholate in an ice-cold condition. Embryos were then stained at room temperature in phosphate-buffered saline containing 5 mM KFe(CN)6, 5 mM K4Fe(CN)6, 1 mg/ml X-gal and 2 mM MgCl2, and rinsed in phosphate-buffered saline. Embryos which expressed blue color were fixed once again as above and stored in methanol.

**RNA extraction and Northern blot analysis**

RNAs were extracted from embryos by Proteinase K-phenol methods, electrophoresed on 1% agarose-formaldehyde gels, and blotted onto Hybond-N membranes (Amersham) (Shinga et al., 1996). Hybridization was carried out under high stringency conditions (0.5 M sodium phosphate buffer, pH 7.2, containing 7% SDS and 1 mM EDTA at 65°C), using 32P-labeled XS3D ORF as a probe (Shinga et al., 1996).

When RNAs were extracted from embryos (10 embryos per sample) injected with SAMDC mRNA, calibration of the amount of the injected mRNA was performed based on the strength of the signals obtained using as references SAMDC mRNA of known amounts (0.05, 0.5, and 5 ng), which had been once mixed with the homogenate of 10 embryos and then extracted therefrom (data not shown).

**Incorporation of radioactive precursors**

Control and SAMDC mRNA-injected embryos were cultured in 1x Steinberg’s solution throughout the experiments. Control embryos were first dissected and dissociated into cells by gently shaking in 0.02 M EDTA in Stearns’ Ca2+-Mg2+-free medium (Shiokawa and Yamana, 1967). SAMDC mRNA-injected embryos were stripped of the vitelline membrane at the early gastrula stage, and the dissociated cells obtained were cultured in complete Stearns’ solution (Shiokawa and Yamana, 1967), containing either 3H-thymidine (1 μCi/ml, 15.1 Ci/mmol), 3H-uridine (1 μCi/ml, 22 Ci/mmol), or 14C-leucine (1 μCi/ml, 303 mCi/mmol) (Amersham) for 3 h.

Cells were collected, homogenized in cold 5% trichloroacetic acid (TCA), and centrifuged at 10,000 rpm for 5 min. Precipitates obtained were suspended in 5% TCA and final precipitates were dissolved in an scintillation mixture (ACSII) to determine radioactivity in a liquid scintillation spectrophotometer.

**Assays for SAMDC activity**

Embryos were homogenized in 50 mM Heps-KOH (pH 7.4), containing 50 mM KC1, 0.1 mM EDTA, 2.5 mM DTT, 10% glycerol and 20 μM FUT175. Soluble fractions, obtained by centrifugation at 16,000 rpm for 10 min, were dialyzed at 4°C against the homogenizing buffer. The activity of SAMDC was assayed by measuring the release of 14CO2 from the reaction mixture containing the homogenate, 2 mM putrescine and 0.2 mM of (carboxyl-14C) SAM (2.6 kBq/mmole) (New England Nuclear), after being incubated at 37°C for 30 min and mixed with an equal volume of 2 M citric acid (Shinga et al., 1996).

**Determination of polyamine and SAM contents**

Embryos were homogenized in 10 mM Tris-HCl buffer (pH 7.5), containing 1 mM DTT, 1 mM EDTA, 20% (W/V) glycerol, and 20 μM FUT175 (10 embryos/400 μl). Polyamines were extracted from homogenates in the TCA-soluble fraction, and analyzed in a high-performance liquid chromatography system using a TSKgel Polyaminepak columns (4.6x50 mm) ( Tosoh) (Shinga et al., 1996). For determination of the amount of SAM and dcSAM, embryos were homogenized in ice-cold 5% TCA (50 embryos/300 μl), and the soluble fraction was obtained by centrifugation at 10,000 rpm for 5 min. The TCA-soluble fraction was analyzed by HPLC according to Seiler and Knodgen (1985), using a TSKgel ODS-80TM column (4.6x150 mm) ( Tosoh). Identification and calibration of SAM and dcSAM were performed using authentic SAM and dcSAM as references.

**Acknowledgments**

We thank Drs. E. Amaya and M. Kirschner for kindly providing us with the Xenopus globin casset plasmid, pSP36T, and Drs. I.B. David and H. Okano for supplying us with the cDNA for rp-1. We also thank Drs. K. Tashiro, M. Wakiyama, H. Fukamachi, and Mr. K. Kondo, T. Muto, and N. Imai for technical help in some of the experiments. Special thanks are due to Drs. A.E. Pegg and T.L. Wilkinson for critical reading of the manuscript.

*Added in proof: We also found that the processing-defective human SAMDC (gift from Dr. A.E. Pegg) overexpressed in Xenopus embryos does not induce the cell dissociation described herein.

**References**


Received: June 1997
Accepted for publication: March 1998