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Level of histone H4 mRNA in *Xenopus laevis* embryonic cells cultured in the absence of cell adhesion

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SUMMARY

The amount of histone H4 mRNA per embryo was followed during early development of *Xenopus laevis* by Northern blot analyses using a cloned histone H4 cDNA as the probe. The H4 mRNA content was nearly constant until the blastula stage, increased greatly at the gastrula stage and then decreased at the neurula stage. Experiments with actinomycin D suggested that most H4 mRNA molecules detected at the late gastrula and neurula stages were maintained depending on new transcription of H4 genes during these stages. To see if the H4 mRNA level is affected by cell adhesion, we prepared dissociated cells and measured H4 mRNA content under conditions that inhibit cellular reaggregation. It was found that the amount of H4 mRNA per embryo in dissociated and reaggregation-inhibited cells was nearly equal to that of the control embryo at the neurula stage. Therefore, we conclude that the synthetic activity of histone H4 mRNA is not dependent on the cellular adhesion during development.

INTRODUCTION

Cell-to-cell interaction is an important process in morphogenesis and it may regulate later gene expression in developing embryos as has been reported in cellular slime moulds (Newell, Longlands & Sussman, 1971), sea-urchin embryos (De Petrocellis & Vittorelli, 1975) and chick embryos (Moscona, Moscona & Saenz, 1968).

In *Xenopus laevis* embryogenesis, several cases have been reported where cellular interaction appears to have an essential function for new gene expression. For example, it is needed in cellular commitment at the midblastula stage, leading to a postgastrular increase in alkaline phosphatase activity (Miyahara, Shiokawa & Yamana, 1982). Also, synthesis of a considerable number of proteins that were directed by new transcripts at the neurula stage appears to depend on the maintenance of cell adhesion during development (Shiokawa *et al.* 1985*a*). The activation of α-actin gene, which is specific for muscle cells, seems to be dependent

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on cellular interaction at the late gastrula stage (Gurdon, Brennan, Fairman & Mohun, 1984; Mohun *et al.* 1984). Furthermore, more recent work by Jones & Woodland (1986) and Sargent, Jamrich & Dawid (1986) revealed that induction-dependent and embryo-region-specific gene expression is also dependent on specific cellular interaction realized in the restricted phase of early embryonic development.

Apart from those genes listed above, however, there are also genes whose expression is not dependent on cellular interaction. For example, as early as 1966 we showed that rRNA genes are expressed independently of cell adhesion by demonstrating equal activation of these genes in the aggregates of dissociated blastula cells of widely differing sizes (Yamana & Shiokawa, 1966; Shiokawa & Yamana, 1967; Miyahara, Shiokawa & Yamana, 1968). Since rRNA genes are typical house-keeping genes, we propose that the expression of other house-keeping genes may also be independent of cell interaction.

In the present experiments, we followed developmental changes in the steady-state amount of histone H4 mRNA and analysed the effects of inhibition of cellular adhesion on the level of histone H4 mRNA during development. The results obtained showed that the amount per embryo of histone H4 mRNA follows a unique temporal change during development and the expression of the histone H4 gene does not appear to depend on the occurrence of cell adhesion during development.

MATERIALS AND METHODS

Embryos and dissociated embryonic cells

Eggs of *Xenopus laevis* were obtained by injection of gonadotropic hormone (Shiokawa & Yamana, 1967) and artificial fertilization was carried out as in Tashiro, Misumi, Shiokawa & Yamana (1983). Embryos were dejellied and cultured until the desired stage (Shiokawa & Yamana, 1967). Dissociation and culture of embryonic cells were carried out using Stearns' medium as described previously (Shiokawa & Yamana, 1967). Staging of embryos was as in Nieuwkoop & Faber (1956).

RNA extraction and purification

Embryos and cells were homogenized in 0·1 M-sodium acetate (pH 5·0) containing 0·5 % sodium dodecyl sulphate (SDS) and 10 μg ml^{-1} of bentonite (Shiokawa & Yamana, 1967). RNA was extracted three times with the buffer-saturated phenol containing 0·1 % hydroxyquinoline, and precipitated from the final aqueous phase with 0·2 M-NaCl and 70 % ethanol.

We compared the efficiency of extraction of H4 mRNA at pH 5·0 (acetate buffer), 7·2 and 9·0 (both with Tris–HCl buffer) by analysing the extracted RNAs by Northern blot methods described below. Efficiency of the extraction was nearly the same with all the procedures, and we used 0·1 M-sodium acetate buffer, pH 5·0, since this buffer facilitated deproteinization most efficiently.

Plasmid

The recombinant plasmid used (a gift from H. R. Woodland) was pcX1H4WI, which carries 382 base pairs of a cDNA clone of histone H4 mRNA from ovary of *Xenopus laevis* at the Bam HI site in the plasmid pAT153. The cDNA clone lacks nucleotides corresponding to three *N*-terminal amino acid residues (Turner & Woodland, 1982). *E. coli* K12 HB101 carrying this

plasmid was cultured in LB medium and plasmid was isolated using the large-scale alkaline method described by Maniatis, Fritsch & Sambrook (1982). When the extracted plasmid DNA was electrophoresed on agarose gel after digestion with Bam HI, both linear pAT153 (approx. 3·6 kb) and histone cDNA fragment (approx. 0·4 kb) were obtained (data not shown).

Nick translation

Nick translation was carried out following the methods of Rigby, Dieckmann, Rhodes & Berg (1977). A reaction mixture (25 μl) contained 50 mM-Tris–HCl (pH 7·5), 10 mM-$MgSO_4$, 1 mM-DTT, 50 μg ml^{-1} BSA, 5 μM each of dATP, dGTP, dTTP, 100 μCi [α-^{32}P]dCTP (410 Ci mmol^{-1}), 50 pg DNase I and 0·5 μg DNA of the total recombinant plasmid pcX1H4WI. The reaction mixture was incubated for 15 min at 37°C, then 1 μl DNA polymerase I (5 units μl^{-1}) (Boehringer–Mannheim) was added and incubation was further continued for 2·5 h at 15°C. The reaction was terminated by adding 75 μl 30 mM-Tris–HCl (pH 8·0) containing 10 mM-EDTA and 10 μg *E. coli* tRNA. Labelled DNA was purified by treatment with chloroform–isoamyl alcohol (1:1) and then precipitated with ethanol. The ^{32}P-labelled plasmid DNA was dissolved in 10 mM-Tris–HCl (pH 8·0) containing 1 mM-EDTA and kept frozen at −20°C until used.

Northern blot hybridization

RNA (up to 10 μg in 8 μl of reaction mixture) was denatured by incubation for 1 h at 50°C in 10 mM-sodium phosphate buffer (pH 7·0) containing 50 % dimethylsulphoxide and 1 M-glyoxal, which had been deionized by the treatment with Bio-Rad AG501(×8) (0·5 g ml^{-1}) for 4 h at 4°C (McMaster & Carmichael, 1977). After being cooled on ice the reaction mixture was mixed with $\frac{1}{4}$ volume of loading buffer containing 50 % glycerol, 10 mM-sodium phosphate (pH 7·0) and 0·4 % bromophenol blue. RNA was electrophoresed at 90 V for 3 h on 1·0 % agarose gel in 10 mM-sodium phosphate buffer (pH 7·0) (Thomas, 1980).

Gels were placed on Whatman 3 MM paper soaked with 20×SSC and overlaid with a nylon filter (Biodyne-A Transfer Membrane, BNNG, 1·2 μm pore size; Pall Ultrafine Filtration Corp., Glen Cove, NY) equilibrated with 3 M-NaCl–0·3 M-trisodium citrate (20×SSC). Transfer was carried out for 15 h as described by Thomas (1980). The filter was baked in a vacuum oven for 1·5 h at 80°C.

Hybridization was carried out essentially following the methods of Wahl, Stern & Stark (1979). Thus, the nylon filter was treated with 20 mM-Tris–HCl (pH 8·0) at 100°C for 5 min to eliminate glyoxal and then treated overnight at 42°C in the hybridization buffer which contained 50 % formamide (deionized as above with AG501(×8) resin), 0·3 % SDS, 0·9 M-NaCl, 50 mM-sodium phosphate buffer (pH 8·3), 5 mM-EDTA, 200 μg ml^{-1} of *E. coli* tRNA and 5 × Denhardt's reagent containing 0·02 % bovine serum albumin, 0·02 % polyvinylpyrrolidone and Ficoll-400. The filter was then hybridized for 20 h at 42°C with ^{32}P-labelled pcX1H4WI (specific radioactivity; 10^8 cts min^{-1} μg^{-1} DNA) which had been previously denatured by boiling for 10 min at 100°C in the above hybridization buffer. The RNA blot was then washed at 50°C with 2×SSC containing 0·1 % SDS, and then with 0·1×SSC containing 0·1 % SDS. Filters were air dried, and exposed to Fuji X-ray film for 2–4 days at −80°C.

RNA was extracted from unfertilized eggs of *Xenopus laevis* at pH 9·0 (Sagata, Shiokawa & Yamana, 1980), and poly(A)$^+$RNA was isolated using a column of poly(U)-Sepharose 4B (Pharmacia) (Shiokawa & Pogo, 1974). When the poly(A)$^-$RNA and poly(A)$^+$RNA were analysed by Northern blotting methods as above, the ^{32}P-signal for histone H4 mRNA was found with poly(A)$^-$RNA but not with poly(A)$^+$RNA (data omitted), in agreement with the previous reports that oocyte-type histone mRNA, but not embryo-type histone mRNA, contains poly(A) sequences (Ruderman & Pardue, 1978; Ruderman, Woodland & Sturgess, 1979; Ballantine & Woodland, 1985).

Throughout experiments, ^{32}P-signals appeared at around 9S–10S in agreement with previous reports (Jacob, Malacinski & Birnstiel, 1976; Ballantine & Woodland, 1985). In the present experiments, hybridization with the vector pAT153 alone was not carried out, since pAT153 (Twigg & Sherratt, 1980) does not cross hybridize with *Xenopus* DNA.

RESULTS

Change in the histone H4 mRNA content during development

Fig. 1 shows the results of Northern blot hybridization of RNAs extracted from normally developing embryos at various stages. The content of histone H4 mRNA remained more or less constant or slightly decreased during cleavage from the unfertilized egg to the blastula stages (lanes 1 to 3). This is consistent with the view that histone H4 mRNA is stored as maternal mRNA before fertilization (Ruderman & Pardue, 1978; Ruderman *et al.* 1979) and is utilized for histone H4 synthesis (Woodland *et al.* 1983). The H4 mRNA content increased greatly at the gastrula stage (lane 4) then decreased at the neurula stage (lane 5).

To confirm that the developmental change in the H4 mRNA content was not due to fluctuation in the recovery efficiency of RNA during extraction, we determined (i) the actual amount (cts min^{-1}) of ^{32}P-label by cutting out each spot from the hybridization filter and (ii) the total absorbancy of the extracted RNA. The radioactivity recovered from the hybridization filter was approximately 1000 cts min^{-1} during cleavage, doubling at the gastrula stage and then dropping to about one-third of the gastrula level at the neurula stage.

When we calculated the specific radioactivity (^{32}P-label per A_{260} unit) for each sample using the actual amount of ^{32}P-label and the total applied optical density of the RNA per lane, we also found that the specific radioactivity increased at the gastrula stage (about 160 % of the average level of cleavage stage) and decreased (to about 60 % of the average level) at the neurula stage (Fig. 2). Therefore, the increase at the gastrula stage as well as the decrease at the neurula stage seem to be reproducible. In fact, such a stage-dependent change as shown in Fig. 1 was repeatedly confirmed in the following experiments. When the embryos reached

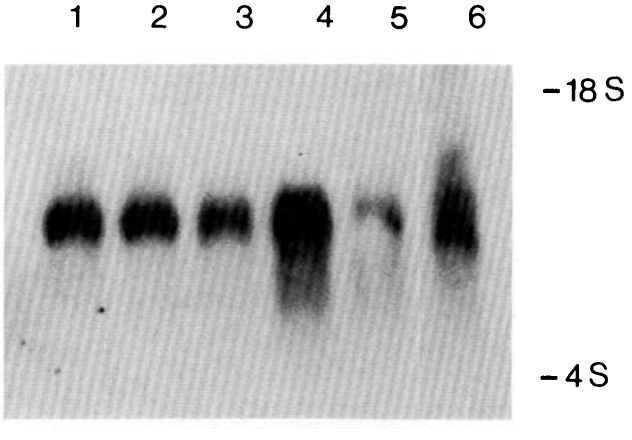


Fig. 1. Northern blot analysis of RNA from whole embryos obtained from the same batch of eggs. RNA from five whole embryos was extracted by phenol treatment at pH 5·0 and analysed by Northern blotting methods using pcX1H4WI as the probe. Lane 1, unfertilized egg; lane 2, stage 3; lane 3, stage 8; lane 4, stage 11; lane 5, stage 17; and lane 6, stage 32. Marker RNAs were denatured and electrophoresed in parallel with the RNAs extracted from the embryos.

the swimming tadpole stage (stage 32) (lane 6), the level of H4 mRNA again increased.

These results strongly suggest that new transcription of histone H4 mRNA occurs at the gastrula stage and that there is some drastic change in the transcription and/or utilization of H4 mRNA during the gastrula and neurula stages.

Change in the histone H4 mRNA content in dissociated cells cultured under reaggregation-inhibited conditions

Two series of experiments were carried out to see if the level of H4 mRNA that is maintained at the gastrula and neurula stages is dependent on cell adhesion during the previous stages. In the first series, embryos were dissociated at the blastula stage and cultured either in the complete Stearns' medium (reaggregation-permitted) or in the Ca^{2+}-free Stearns' medium (reaggregation-inhibited) until the time when sibling embryos reached the gastrula or neurula stage. In the Ca^{2+}-free medium, cellular reaggregation does not take place at all (photographs omitted, but see Miyahara *et al.* 1982; Shiokawa *et al.* 1985*a*), but cells continue to divide and synthesize rRNA normally (Miyahara *et al.* 1982), although there will be significant inhibition in protein synthesis (Shiokawa *et al.* 1985*a*).

Fig. 3 shows the H4 mRNA levels determined under the two different culture conditions when the cells had reached the equivalent of gastrula or neurula stages. It is apparent that in neither case was there a significant difference in the ^{32}P-signal between the whole embryos (lanes 1 and 2), the cultures of reaggregating cells (lanes 3 and 4) and the cultures of reaggregation-inhibited cells (lanes 5 and 6). These results show that the histone H4 mRNA level in the gastrula and neurula cells is not affected at all by the presence or absence of prior cellular adhesion at least in the postblastular stages.

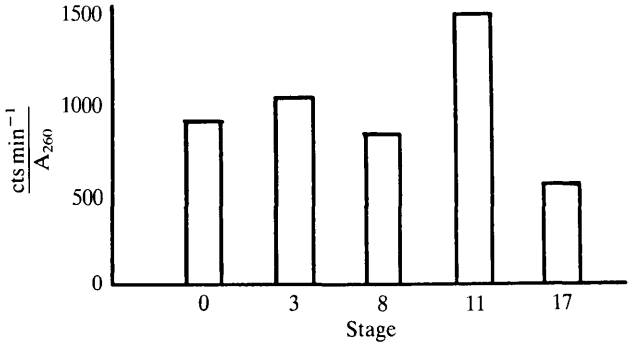


Fig. 2. Change in the amount of ^{32}P-signal hybridized to H4 mRNA. Spots showing ^{32}P-signal were cut out from the Northern blot filter obtained in the experiment in Fig. 1, and ^{32}P-radioactivity was counted in ACS scintillation mixture in a scintillation spectrometer. The amount (A_{260}) of RNA applied onto each gel slot was determined and the ^{32}P-amount was normalized using the A_{260} absorption of the RNA used. Since the total amount of RNA per embryo does not change greatly depending on the stages examined, the amount of the ^{32}P-label plotted represents a direct measure of the H4 mRNA detected per embryo at each stage.

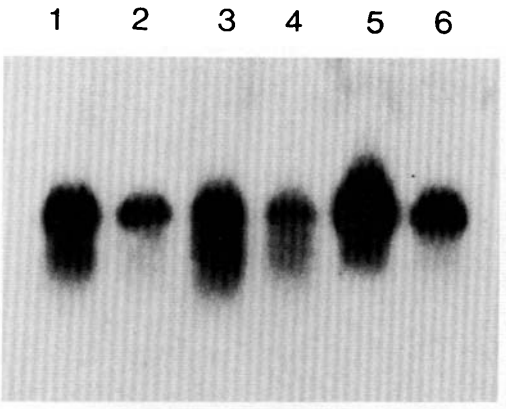
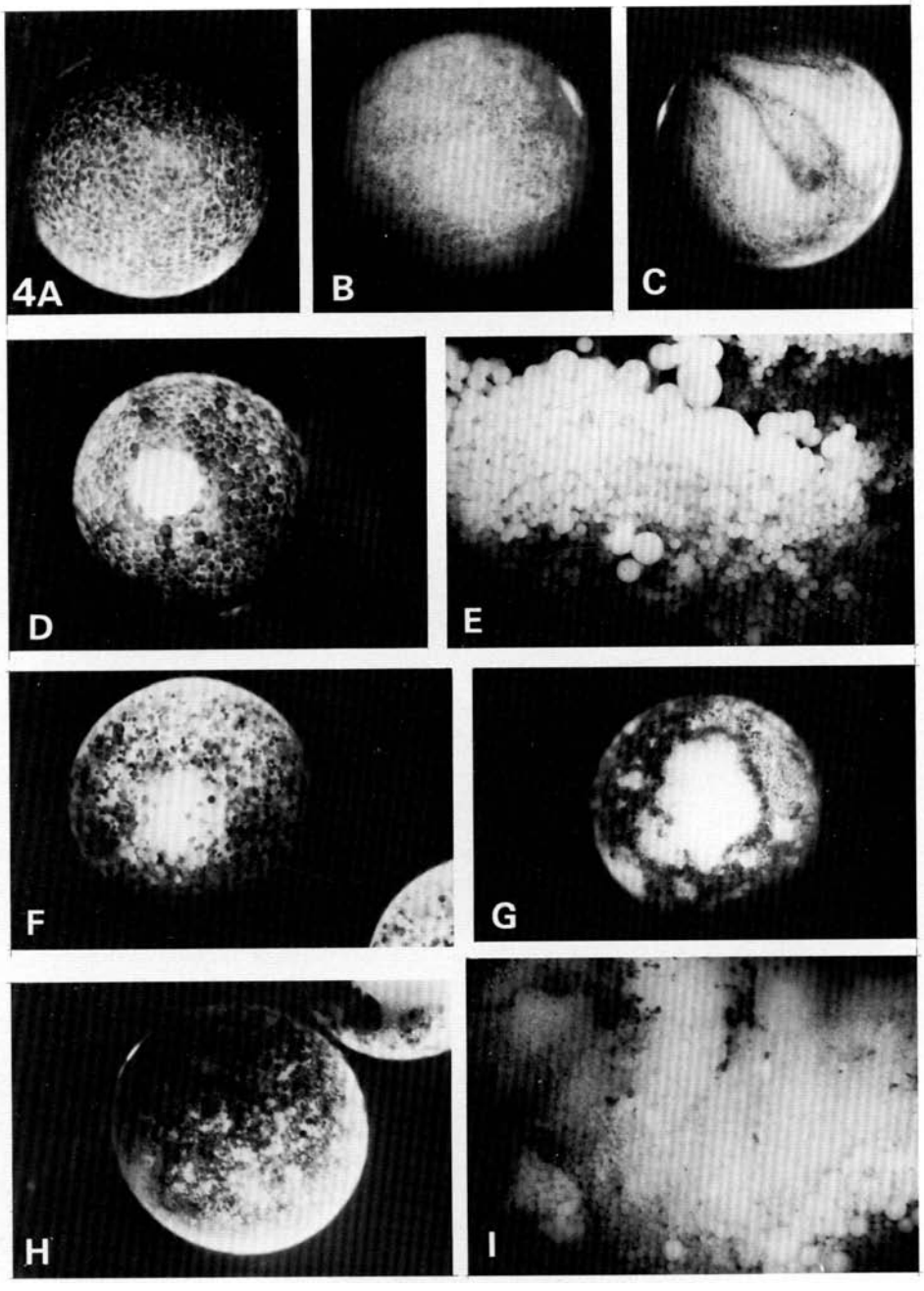


Fig. 3. Northern blot analysis of RNAs extracted from whole embryos and dissociated cells treated in the reaggregating and non-reaggregating medium. Lanes 1 and 2 are for control embryos. Embryos for experiment were dissociated at the blastula stage and then cultured either in complete Stearns' medium (lanes 3 and 4) which permitted reaggregation or in the Ca^{2+}-free medium (lanes 5 and 6) which prevented cellular reaggregation. The culture continued until either the gastrula stage (stage 11) (lanes 1, 3 and 5) or the neurula stage (stage 17) (lanes 2, 4 and 6). RNAs extracted from each of the cultures (equivalent to three embryos) were analysed as in Fig. 1.

In the second series of experiments, uncleaved fertilized eggs were cultured in 80 mM-phosphate buffer shortly after fertilization (Takeichi, Satoh, Tashiro & Shiokawa, 1985; Shiokawa *et al.* 1985*b*). When embryos were cultured in 80 mM-phosphate buffer (pH 7·6), cell adhesion among the blastomeres was inhibited from as early as the 2- and 4-cell stages (photographs omitted, but see Takeichi *et al.* 1985; Shiokawa *et al.* 1985*b*). Fig. 4D shows the treated embryo at the blastula stage. It was apparent that, since the connection between blastomeres was loosened, each blastomere became spherical and blastomeres at the top region of the animal pole dropped down into the blastocoel (Fig. 4D). When we removed the vitelline membrane from such an embryo, blastomeres separated without any further dissociation procedure as shown in Fig. 4E. Therefore, the inhibition of cell adhesion was apparent under these conditions.

When embryos were kept cultured in the phosphate buffer until the gastrula stage, cell division appeared to have become slightly abnormal, as can be seen by the occurrence of relatively larger sizes of blastomeres in such embryos (Fig. 4F).

Fig. 4. Appearance of embryos or cells cultured under various conditions. (A) Control blastula (stage 8); (B) control gastrula (stage 11); (C) control neurula (stage 16). (D) Cultured in 80 mM-phosphate buffer from the uncleaved fertilized egg to the blastula stage. Because cellular adhesion was inhibited, cells at the animal pole region dropped down into the blastocoel. (E) When vitelline membrane of embryos in D was removed the constituent cells were found to lack adhesion. (F) Embryos in D were maintained in the 80 mM-phosphate buffer until the gastrula stage. Some blastomeres appeared to be a little larger than normal ones shown in B. (G) The embryo in D was transferred into Ca^{2+}-free Stearns' medium at the blastula stage and then cultured until the time when the control embryo reached the gastrula stage. (H) Same as in G but culture was continued until the time when the control embryo reached the neurula stage as in C. (I) When the vitelline membrane was removed, the embryo in H was found to be composed of cells that lacked adhesion.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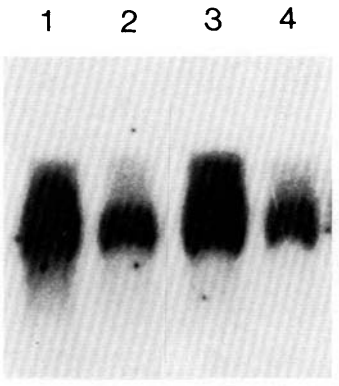

Fig. 5. Northern blot analysis of RNA obtained from embryos cultured under conditions that inhibited cell adhesion. Fertilized eggs were immediately immersed in the 80 mM-phosphate buffer (lanes 3 and 4). Control embryos were cultured in the normal 1/10 Steinberg medium (lanes 1 and 2). Embryos in 80 mM-phosphate buffer (lanes 3 and 4) were transferred into Ca^{2+}-free Stearns' medium at the time when the control embryo reached the blastula stage. Embryos were sacrificed either at the time when the control embryos reached the gastrula stage (stage 11) (lanes 1 and 3) or at the neurula stage (stage 16) (lanes 2 and 4). RNAs equivalent to three embryos were analysed as in Fig. 1.

Therefore, we transferred the phosphate-buffer-treated embryos to the Ca^{2+}-free Stearns' medium at the blastula stage and further cultured the embryos until the time when the control embryos reached the gastrula (Fig. 4G) and neurula (Fig. 4H) stages. Under these conditions, invagination did not take place, another sign of the absence of normal cell adhesion. However, it appeared that the blastomeres of these embryos have continued to divide and become similar in size to those of the control at the gastrula (Fig. 4B) and neurula (Fig. 4C) stages. At the end of culture, by removal of their vitelline membrane (see above; Fig. 4I), the constituent cells of these treated embryos were shown to have separated.

When RNA was analysed in the embryos treated with phosphate buffer and then with the Ca^{2+}-free Stearns' medium, there was no difference in the level of H4 mRNA per embryo between the control and the treated embryos either at the gastrula or neurula stages (Fig. 5). Since the vitelline membrane was not removed from the cleavage embryos in phosphate buffer, there might have been some kind of very weak cell interaction due to the occasional cellular attachment during cleavage (cf. Sargent *et al.* 1986). However, the interaction could not be normal, since the blastomeres were not connected to each other as seen after removal of the vitelline membrane. Therefore, it can be concluded that embryonic cells do not lose the ability to maintain the normal level of H4 mRNA even when normal cell adhesion is disturbed from the very beginning of development.

DISCUSSION

The use of H4 cDNA clone enabled us to show that the level of the histone H4 mRNA in *Xenopus* embryos increased greatly at the gastrula stage and then

decreased at the neurula stage. To see if the increase at the gastrula stage depends on new transcription, we followed the amount of H4 mRNA under the chase conditions with actinomycin D. Thus, cells were dissociated at the pigment gastrula (stage 9·5), late gastrula (stage 11) or neurula (stage 20) stage and then treated with 10 μg ml^{-1} of actinomycin D for 50 min and 150 min, so that the level of H4 mRNA could be followed after the inhibition of RNA transcription (Shiokawa & Yamana, 1967; Shiokawa, Tashiro, Oka & Yamana, 1983). When [^{3}H]uridine was added to the culture under these conditions, it was confirmed that while pulse-labelled 40S pre-rRNA was chased into 28S and 18S rRNA completely, at least one-half of the heterogeneous mRNA-like RNA decayed during the chase of 150 min (data not shown). During the chase period, more than a half level of H4 mRNA disappeared. Therefore, it appeared that the majority of H4 mRNA in the early gastrula stage is metabolically unstable and did not stay until the gastrula stage. This means that most of the H4 mRNA molecules detected at the gastrula and neurula stages were those newly synthesized in these stages.

The new transcription of H4 genes at the gastrula stage is consistent with the reported activation of other histone genes at the same stage (Woodland *et al.* 1983). In this connection, we have previously shown that at least 40 % of the mass of the gastrula poly(A)$^+$mRNA is already newly synthesized mRNA (Shiokawa, Misumi & Yamana, 1981). Furthermore, Woodland, Flynn & Wyllie (1979) have reported that maternal histone H1 mRNA is exhausted already by the gastrula stage.

In the experiment in which embryos were dissociated at the blastula stage, both reaggregation-permitted and reaggregation-inhibited cells maintained the normal levels of H4 mRNA at the gastrula and neurula stages. These results show very clearly that direct cell adhesion is not necessary for the maintenance of the normal level of H4 mRNA in either the neurula or gastrula cells.

In the experiment in which embryos were cultured in phosphate buffer shortly after fertilization, cells were kept separated from the beginning of development. When these embryos were transferred into the Ca^{2+}-free Stearns' medium and assayed for the H4 mRNA level at the gastrula and neurula stages, it was also found that they still maintained the normal level of the mRNA at both stages. Therefore, it can be concluded that the level of H4 mRNA in the cells of gastrulae and neurulae does not depend on the occurrence of cell adhesion during the preceding stages. Since most of the H4 mRNA present in the gastrula and neurula cells is assumed to be newly synthesized as discussed above, these results imply that the maintenance of the transcriptive activity of H4 histone mRNA is not dependent on cell adhesion during development.

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