

Development of translationally active mRNA for larval muscle acetylcholinesterase during ascidian embryogenesis

(egg cytoplasmic determinants/*Xenopus* oocytes/transcriptional control/maternal mRNA/*Ciona intestinalis*)

THOMAS H. MEEDEL AND J. R. WHITTAKER

Boston University Marine Program, Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Communicated by James D. Ebert, May 5, 1983

ABSTRACT Relative quantities of translationally active acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7) mRNA present at various developmental stages were compared in embryos of the ascidian *Ciona intestinalis*. Purified RNA was tested for its translational capacity by microinjection into *Xenopus laevis* oocytes; the acetylcholinesterase produced was immunoprecipitated with antibody to *Ciona* acetylcholinesterase and enzyme activity was assayed radiometrically. With this protocol, enzyme synthesis was found to be directly related to the amount of RNA injected and to the oocyte incubation time. A functional template for acetylcholinesterase was first detected at 6 hr of development (late gastrula) and is probably present as early as 5 hr. The level of this template activity increased until the middle tail formation stage (11–12 hr after fertilization) and then remained constant until 16 hr of development (the final stage examined), 2 hr before hatching. These findings, and the results of previous actinomycin D inhibition experiments, indicate that mRNA for ascidian larval muscle acetylcholinesterase is first synthesized during gastrulation.

Ascidian embryos (subphylum Urochordata, class Ascidiacea) possess an acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7) that is an excellent marker of larval muscle differentiation. The enzyme is first seen histochemically in muscle rudiments of the ascidian neurula stage (1–3) and does not occur in other tissues at prehatching stages of embryonic development (4). Quantitative measurements reveal a very large increase in enzyme activity during the time from neural plate formation to hatching, an increase that is sensitive to inhibitors of protein and RNA synthesis (4). The results of various studies, both classical and recent, suggest a role for egg cytoplasmic determinants in regulating the occurrence of this acetylcholinesterase as well as other features of larval muscle differentiation (5). Especially compelling evidence for a determinative role of cytoplasmic factors is the otherwise anomalous development of acetylcholinesterase in nonmuscle cells into which muscle lineage cytoplasm was experimentally introduced (6, 7).

A discrete time period seems to exist in which RNA synthesis is required for larval acetylcholinesterase development. Actinomycin D treatment begun before early gastrulation prevents enzyme formation but has less effect on the amount of enzyme activity produced the later it is added, finally causing no response when added after the middle tail formation stage (4, 8). One likely interpretation of this finding and other actinomycin D results with ascidians (2, 9, 10) is a transcription of the muscle acetylcholinesterase gene between gastrulation and the middle tail formation stages.

Most features of muscle determinant behavior in ascidians could be explained adequately by the hypothesis that egg cy-

toplasmic determinants are simply preformed maternal mRNAs for the proteins involved in the various histospecific expressions of mosaic development. Although the studies with actinomycin D suggest otherwise, one is reluctant to accept the simple interpretation of the results of inhibitor experiments, especially given their limitations as essentially negative observations. To investigate the question of preformed maternal mRNA in particular, and to lay the groundwork for a more general understanding of the regulation of muscle acetylcholinesterase in development, we assayed translationally active acetylcholinesterase mRNA by microinjecting ascidian RNA into *Xenopus laevis* oocytes. Soreq *et al.* (11) have used such an assay in *Xenopus* oocytes to study acetylcholinesterase mRNA in rat brain and *Torpedo* electric organ RNA preparations. We modified their technique to include an immunoprecipitation step with antibody prepared against purified ascidian acetylcholinesterase (12). Translationally active acetylcholinesterase mRNA was not found in RNA prepared from embryos before the 64-cell stage, but it was present in increasing amounts in RNA isolated from embryos between late gastrula and middle tail formation stages.

MATERIALS AND METHODS

Ciona intestinalis Linnaeus was collected near Woods Hole, MA. Eggs were obtained surgically from the oviducts and fertilized artificially, and embryos were raised in Millipore-filtered sea water at $18 \pm 0.1^\circ\text{C}$. In the experiments reported, normal development occurred in more than 95% of the embryos.

RNA was prepared from embryos of various developmental stages by extraction with phenol/chloroform (13). Any remaining protein was digested with proteinase K (14); DNA and tRNA were removed by washing the RNA twice with sodium acetate (14). Purified RNA was dissolved in water at concentrations of 5–10 mg/ml, divided into aliquots, and stored at -85°C .

Female *Xenopus laevis* were purchased from NASCO (Fort Atkinson, WI). Stage six oocytes (15) were dissected free of smaller oocytes and follicle cells and stored at 18°C in modified Barth's medium (16). RNA was microinjected into oocytes in volumes of 25 or 50 nl; control oocytes were injected with the corresponding amount of distilled water. Groups of 5–10 oocytes were incubated at 18°C for the desired times in 100 μl of modified Barth's medium. This assay for acetylcholinesterase mRNA devised by Soreq *et al.* (11) takes advantage of the high efficiency of this translation system (17) and the ability of frog oocytes to synthesize biologically functional proteins on exogenously added mRNA (18).

Acetylcholinesterase activity was measured in samples (either oocytes or incubation medium) by the method of Schrier *et al.* (19). In order to prepare samples for enzyme assay, the incubation medium was removed, and the oocytes were homoge-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

nized in 100 μ l of 50 mM Tris·HCl, pH 7.2/145 mM NaCl/2% Triton X-100/1% sodium deoxycholate. After 30 min on ice with intermittent mixing, the oocyte homogenates were centrifuged at 12,000 \times g for 5 min, and the supernatant between the floating lipid layer and the melanin-containing residue was removed. The remaining material was reextracted and the two supernatants were combined. Acetylcholinesterase was isolated from samples by adding antiserum (IgG) to *Ciona* acetylcholinesterase (12) and using formalin-fixed *Staphylococcus aureus* (Immuno-Precipitin, Bethesda Research Laboratories) to recover the enzyme-antibody complexes (20). Because this antiserum binds to acetylcholinesterase without affecting catalytic activity, it can be used to purify and quantitate functional enzyme. The insoluble complexes ("immunoprecipitates") were washed three times with 50 mM Tris·HCl, pH 7.2/145 mM NaCl/0.5% Triton X-100 and resuspended in 40 μ l of 62.5 mM potassium phosphate, pH 7.4/1.25 mM EDTA/250 mM NaCl/0.625% Triton X-100. Ten microliters of [*acetyl*- 14 C]acetylcholine iodide (4 mCi/mmol; 1 Ci = 3.7×10^{10} Bq) in water was added to give a final concentration of 0.3 mM, and the samples were incubated with constant agitation at 21°C for the times stated. The amount of acetylcholine hydrolyzed by immunoprecipitates of oocytes injected with water was subtracted from all experimental samples. This level (less than 1% of the substrate in the assay) was about the same as the spontaneous hydrolysis of acetylcholine under standard conditions. With larval acetylcholinesterase, enzyme activity was linear for at least 40 hr if less than 25–30% of the substrate was hydrolyzed.

RESULTS

Oocytes of *Xenopus laevis* produce enzymatically active acetylcholinesterase when injected with appropriate mRNAs (11). By combining the oocyte protein synthesis system and antiserum to *Ciona intestinalis* acetylcholinesterase, we examined changes in the level of translatable mRNA for this enzyme during ascidian development. The immunologic procedure was specific for the *Ciona* enzyme; the antiserum did not recognize endogenous oocyte cholinesterases (Table 1). When quantities of crude *Ciona* acetylcholinesterase in amounts similar to the levels detected in microinjected oocytes (see below) were mixed with oocyte extracts more than 80% of the ascidian enzyme was specifically immunoprecipitated (Table 1). Increasing the quantity of acetylcholinesterase by 20 times yielded immunoprecipitates with 60% of the original enzyme activity. This small variation

Table 1. Characterization of antiserum to *Ciona* acetylcholinesterase

Sample	Additions, μ l				Acetylcholine hydrolyzed, μ mol $\times 10^3$
	<i>Xenopus</i> extract	<i>Ciona</i> acetylcholinesterase	Immune IgG	Pre-immune IgG	
1	90	10	5	—	2.50
2	90	10	—	5	0.10
3	90	—	5	—	0.03

After incubation with the indicated IgG, all samples were allowed to react with *S. aureus*; the resulting complexes were washed and assayed for 24 hr to determine immunoprecipitated acetylcholinesterase activity. Crude acetylcholinesterase was prepared from 20-hr *Ciona* larvae as described (4). Ten microliters of this enzyme preparation hydrolyzed 3×10^{-3} μ mol of acetylcholine during the assay period. Extracts of *Xenopus* oocytes were prepared as described in the text; the cholinesterase activity present in 10 μ l of this extract (equivalent to one oocyte) hydrolyzed all of the substrate (16×10^{-3} μ mol of acetylcholine) during the time of assay. Spontaneous hydrolysis of acetylcholine during the reaction period (0.08×10^{-3} μ mol) was subtracted from the values for all samples.

in the percentage of acetylcholinesterase recovered demonstrates that the immunological protocol, although not completely quantitative as used, is suitable for making comparisons over a wide range of enzyme concentrations.

RNA from 17-hr embryos was used for initial injection studies, because earlier work indicated that acetylcholinesterase mRNA is present at this time of development (2, 4). Template for acetylcholinesterase was readily detected in this RNA, although none of the enzyme produced was found in the incubation medium (Table 2). Because secretory proteins synthesized on exogenous mRNAs are usually exported by *Xenopus* oocytes (18), this result may mean that ascidian larval muscle acetylcholinesterase is not normally secreted *in vivo*. Retention of the enzyme by oocytes also reinforces the importance of using the antiserum to eliminate contamination with endogenous cholinesterases.

To accomplish our objective of comparing relative amounts of acetylcholinesterase mRNA at different stages of embryogenesis, assay conditions were established that resulted in linear enzyme production with respect to the amount of RNA injected per oocyte and to the length of time that injected oocytes were incubated. RNA from 17-hr embryos was used for these measurements. Results with oocytes from two frogs revealed increasing acetylcholinesterase production up to approximately 300 ng of RNA per oocyte; higher concentrations were slightly inhibitory (Fig. 1A). Injecting 60–80 ng of RNA per oocyte resulted in linear synthesis of acetylcholinesterase for at least 45 hr (Fig. 1B). On the basis of these experiments one can define conditions that generally result in a linear response when various amounts of RNA from different stages of development are injected. For all such studies RNA concentrations of 15–50 ng per oocyte were used, and incubations were for 10–20 hr. Each experiment was done at least twice with oocytes from a single frog then repeated with oocytes from a second frog. Results are expressed on a RNA basis because synthesis of rRNA (which represents almost all the RNA of our preparations) is not detected until after hatching (13).

In *Ciona*, a constant low level of acetylcholinesterase occurs throughout early development and up to the late gastrula stage (6 hr). The activity of this enzyme rises from 7 hr of development until after the time of hatching at 18 hr after fertilization (4). The first part of this increase (before 11–12 hr) is sensitive to actinomycin D, suggesting a role for mRNA synthesis in the accumulation of acetylcholinesterase (4, 8). If our interpretation is correct, no translatable mRNA for acetylcholinesterase is present before 6–7 hr of development; afterwards such mRNA should occur. This prediction was tested by isolating RNA from embryos at different times of development and assaying the RNA for its capacity to direct synthesis of ace-

Table 2. Acetylcholinesterase production by microinjected oocytes

Exp.	Acetylcholine hydrolyzed, μ mol $\times 10^4$ /25 ng RNA per oocyte		
	Oocyte extract	Incubation medium	% secreted
1	10.9	0.3	3
2	1.7	0.05	3

Oocytes microinjected with RNA from late tail formation stage *Ciona* embryos were incubated for 12 hr. Enzyme activity in immunoprecipitates of the incubation medium or oocyte extracts was assayed for 15 hr (Exp. 1) or 10 hr (Exp. 2). Oocytes from different frogs were used in the two experiments, which may account for the variation in enzyme production.

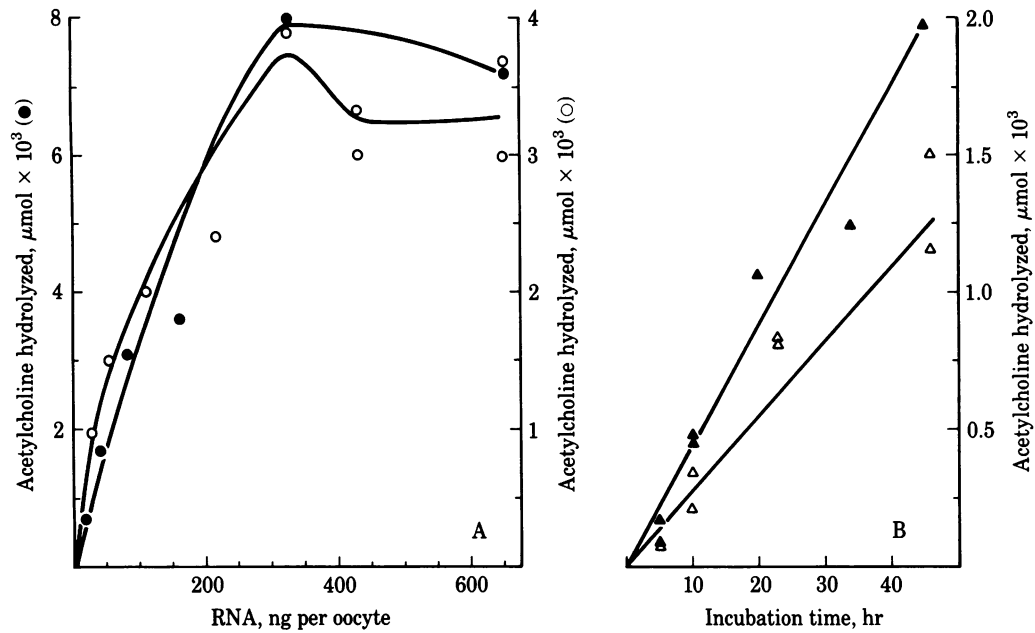


FIG. 1. Characterization of acetylcholinesterase production by oocytes microinjected with RNA from 17-hr *Ciona* embryos. (A) Acetylcholinesterase production as a function of the amount of injected RNA. In the first experiment (●) microinjected oocytes were incubated for 20 hr and immunoprecipitated enzyme was assayed for 16 hr. In the second experiment (○) microinjected oocytes were incubated for 12 hr and the immunoprecipitated enzyme was assayed for 16 hr. Each point represents acetylcholinesterase produced by five oocytes. (B) Acetylcholinesterase production as a function of oocyte incubation time. Oocytes were injected with RNA and incubated for the indicated times. In the first experiment (▲) each oocyte was injected with 82 ng of RNA and enzyme assays were done for 20 hr. In the second experiment (△) each oocyte was injected with 66 ng of RNA, and enzyme assays were done for 8 hr. Each point represents acetylcholinesterase produced by five oocytes.

tylcholinesterase in the oocyte translation system (Fig. 2A). No mRNA for acetylcholinesterase was detected at 4 hr of development, even when 300 ng of RNA was injected per oocyte. By 8 hr after fertilization such template activity was found, and its level was approximately 2/3 that seen in RNA from 12-hr embryos. At 16 hr of development a slight decrease in the amount

of acetylcholinesterase mRNA was noted. A changing level of translatable acetylcholinesterase mRNA seems to accompany the increase in activity of this enzyme from 8–12 hr of development. Continued accumulation of enzyme activity after 12 hr may result from translation of a fairly stable population of mRNA.

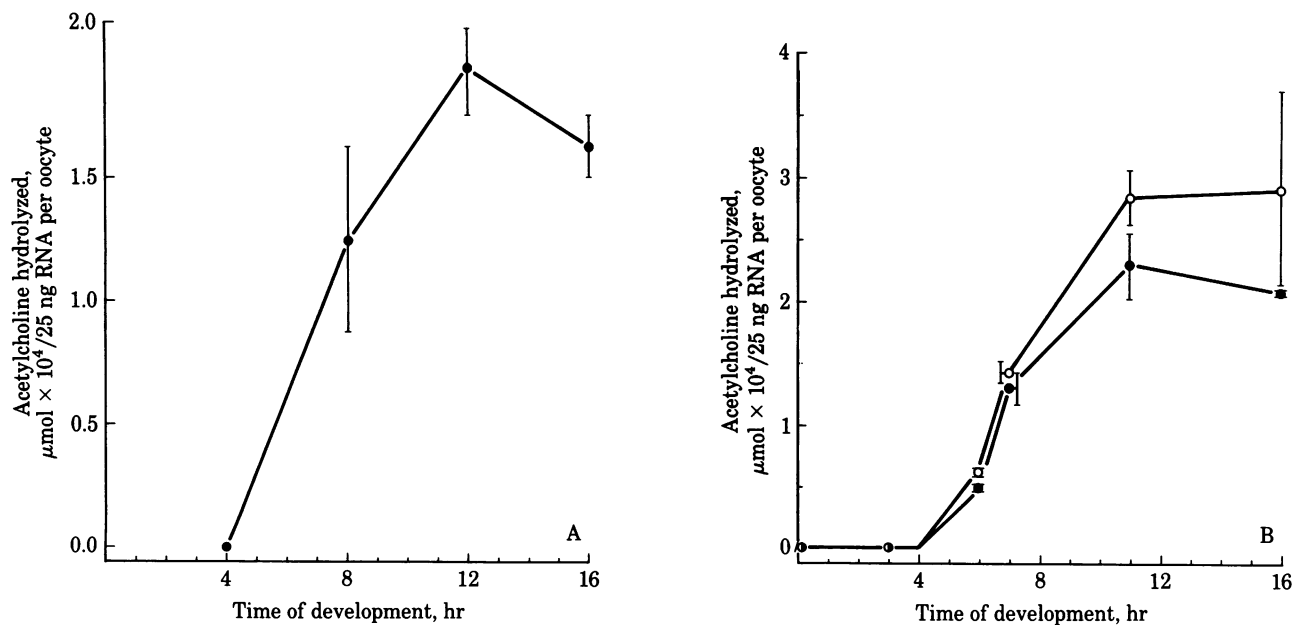


FIG. 2. Accumulation of translatable acetylcholinesterase mRNA in developing *Ciona* embryos. (A) RNA was prepared from embryos at the times indicated and injected into oocytes from a single frog. Incubation was for 15 hr and immunoprecipitated acetylcholinesterase was assayed for 12 hr. For each point RNA was injected at two concentrations (20 or 40 ng per oocyte); the bars represent the range in the quantities of acetylcholinesterase produced. (B) RNA was prepared from embryos at the times indicated and injected into oocytes from two frogs. Oocytes from one frog were incubated for 12 hr and immunoprecipitated enzyme was assayed for 12 hr (●). With oocytes from a second frog, corresponding periods were 12 hr and 15 hr (○). For each point, two groups of eight oocytes were injected; the bars represent the range in the quantities of acetylcholinesterase produced.

The relative quantity of translatable acetylcholinesterase mRNA at various developmental stages was examined in a second group of embryos (Fig. 2B). As expected from the previous experiment, unfertilized eggs and 3-hr embryos had no detectable acetylcholinesterase mRNA. By 6 hr of development a substantial population of a mRNA was found. This template activity increased between 6 and 7 hr and between 7 and 11 hr of development but was stable between 11 and 16 hr. The results of these two sets of measurements are consistent and they imply that translatable mRNA for acetylcholinesterase first appeared between 4 and 6 hr after fertilization.

The apparent absence of acetylcholinesterase mRNA from unfertilized eggs and pregastrula stage embryos might simply indicate degradation of the mRNA during RNA isolation from the early stages. Selective degradation seems an unlikely explanation because RNA from all stages was prepared in the same way and because three series of isolates behaved similarly. Nonetheless, we examined this question further by using RNA from 0-hr, 3-hr, and 16-hr embryos as templates in a mRNA-dependent rabbit reticulocyte lysate assay (21); all efficiently directed the synthesis of high-molecular weight polypeptides.

We have also considered the possible occurrence of factors in our pregastrula stage RNA preparations that might prevent

translation of acetylcholinesterase mRNA in the *Xenopus* oocyte. When 0-hr or 3-hr RNA samples were mixed with 16-hr RNA before microinjection into the oocytes, no suppression of the subsequent translation of ascidian acetylcholinesterase from the 16-hr RNA was found.

DISCUSSION

Xenopus laevis oocytes are suitable as an *in vivo* protein synthesis system for examining the level of translationally active acetylcholinesterase mRNA during ascidian development. A sensitive radiometric assay was used to quantitate the amount of enzyme produced. We modified the method of Soreq *et al.* (11) by purifying the reaction products with antiserum to *Ciona* acetylcholinesterase; this procedure eliminated oocyte-associated cholinesterases and ensured that only the ascidian enzyme was measured. Because the quantity of enzyme produced was a linear function of the amount of RNA injected and the immunological methods yielded nearly the same percentage of recovery over wide ranges of enzyme activity, we feel that the levels of translationally active acetylcholinesterase mRNA determined for different stages of development correspond to their relative levels *in vivo*.

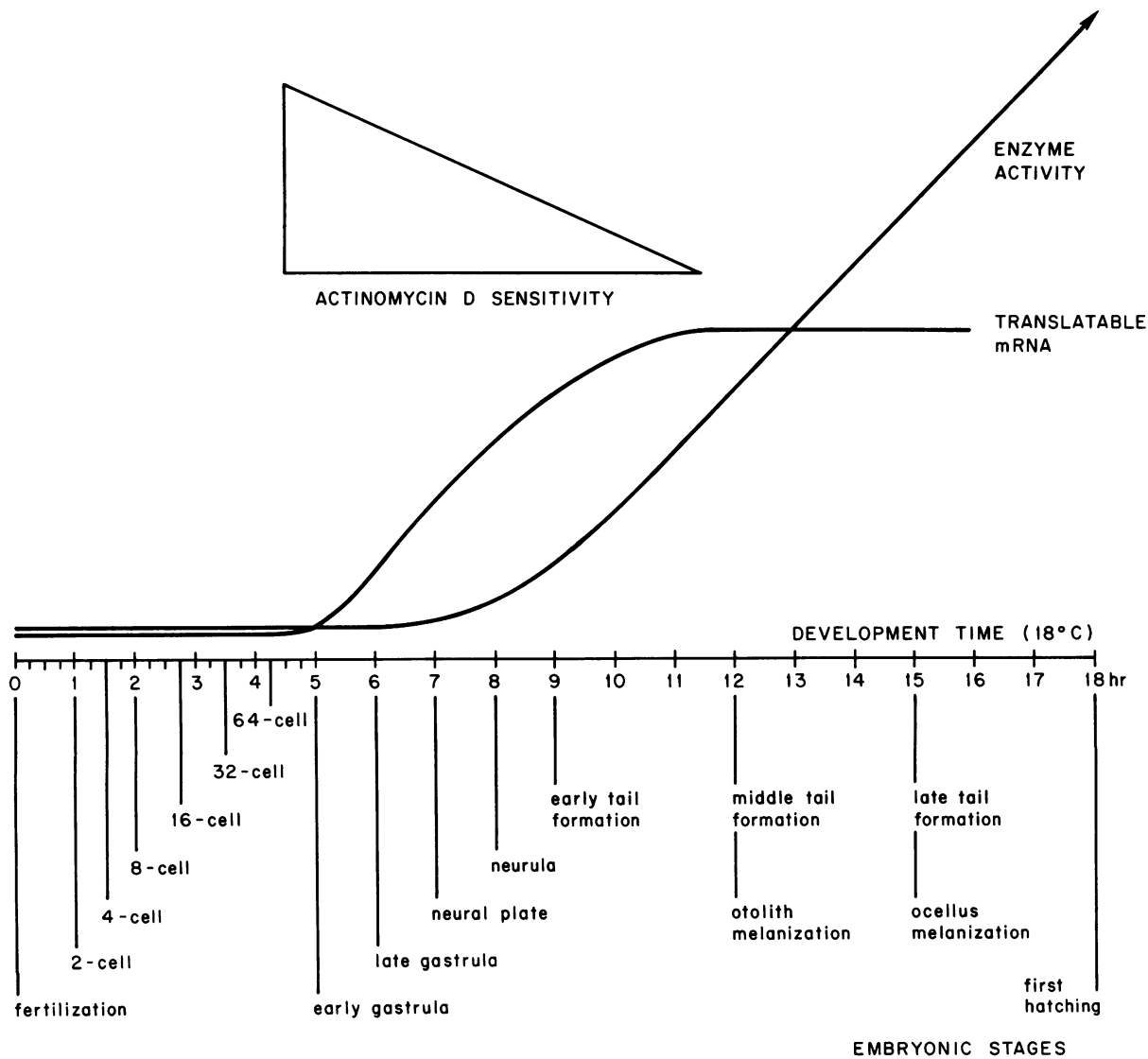


FIG. 3. Diagram relating the events concerned with acetylcholinesterase development to the embryonic stages of *Ciona intestinalis*.

The events related to acetylcholinesterase expression in this and previous studies are summarized in Fig. 3. Our present results reveal the appearance and accumulation of translatable acetylcholinesterase mRNA during ascidian embryogenesis. This mRNA was first detected at 6 hr of development; however, by extrapolating the results of our experiments to zero acetylcholinesterase mRNA, we estimate that this template first appears about 5 hr after fertilization (early gastrula stage). Until 11–12 hr of development accumulation of the mRNA paralleled the rising enzymatic activity of acetylcholinesterase observed previously (4). Afterwards, the quantity of mRNA leveled off as enzyme activity continued increasing.

Actinomycin D must be added to cultures of embryos before 5 hr of development to block completely the increase in acetylcholinesterase activity (8). Because this is 3 hr before the first detectable increase in enzyme activity (4, 8), some regulation at the translational level may exist. The occurrence of translatable acetylcholinesterase mRNA at 6 hr of development (and probably beginning at 5 hr) supports this possibility. Even if such regulation does occur, similarities between the ontogenies of enzyme activity and translatable mRNA indicate that the most important control involves the accumulation of a functional mRNA.

The results of the present study, together with previous work on the effects of actinomycin D, offer a consistent view of acetylcholinesterase mRNA synthesis occurring from early gastrula to middle tail formation stages and support the idea of a localized regulatory factor operating at the level of gene transcription. The egg cytoplasmic determinant that regulates expression of acetylcholinesterase in larval muscle seems unlikely to be a maternally preformed mRNA for the enzyme.

While our findings rule out the existence of an inactive maternal proenzyme or masked (by protein) maternal mRNA as the cytoplasmic factor for acetylcholinesterase, they do not exclude chemical modification of an existing, nonfunctional mRNA to a translationally active form (22, 23). In addition to transcriptional control, which we consider to be the most likely explanation of our results, other kinds of nuclear regulatory mechanisms such as processing are also possible. Regardless of its exact nature, any such event must presumably be sensitive to actinomycin D.

The processes limiting further accumulation of translatable acetylcholinesterase mRNA after 11–12 hr of development are also unknown. Because increased enzyme activity is not prevented in embryos exposed to actinomycin D after this stage (4, 8), *de novo* mRNA synthesis probably stops at this time. Increased acetylcholinesterase activity would then depend on a stable mRNA population.

The assay method we describe for measuring translationally active acetylcholinesterase mRNA has important advantages of specificity and directness over the previous actinomycin D ex-

periments used to identify transcriptionally active periods in ascidian development. Results of our assays eliminate much of the ambiguity in interpreting the negative results of inhibitor experiments, and in so doing support the conclusion drawn from those earlier studies that acetylcholinesterase is regulated by transcriptional level controls beginning during gastrulation. A more direct and precise description of transcriptional activity and its timing will be possible by using DNA complementary to the acetylcholinesterase mRNA. In particular, use of such probes could resolve the still open question of a translationally inactive maternal mRNA. Our assay can be an aid to procedures for screening cDNA or genomic libraries for the appropriate complementary sequences.

We thank Dr. John O'Loughlin for a gift of reticulocyte lysate. This research was supported by National Science Foundation Grant PCM 79-04210 and Grant HD 09201 from the National Institute of Child Health and Human Development.

1. Durante, M. (1956) *Experientia* **12**, 307–308.
2. Whittaker, J. R. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 2096–2100.
3. Ohmori, H. & Sasaki, S. (1977) *J. Physiol. (London)* **269**, 221–254.
4. Meedel, T. H. & Whittaker, J. R. (1979) *J. Exp. Zool.* **210**, 1–10.
5. Whittaker, J. R. (1979) in *Determinants of Spatial Organization*, eds. Subtelny, S. & Konigsberg, I. R. (Academic, New York), pp. 29–51.
6. Whittaker, J. R. (1980) *J. Embryol. Exp. Morphol.* **55**, 343–354.
7. Whittaker, J. R. (1982) *Dev. Biol.* **93**, 463–470.
8. Meedel, T. H. (1983) *J. Exp. Zool.*, in press.
9. Whittaker, J. R. (1979) *Biol. Bull.* **157**, 344–355.
10. Satoh, N. (1979) *J. Embryol. Exp. Morphol.* **54**, 131–139.
11. Soreq, H., Parvari, R. & Silman, I. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 830–834.
12. Meedel, T. H. (1980) *Biochim. Biophys. Acta* **615**, 360–369.
13. Meedel, T. H. & Whittaker, J. R. (1978) *Dev. Biol.* **66**, 410–421.
14. Lee, D. C., McKnight, G. S. & Palmiter, R. D. (1978) *J. Biol. Chem.* **253**, 3494–3503.
15. Dumont, J. N. (1972) *J. Morphol.* **136**, 153–179.
16. Gurdon, J. B. (1977) in *Methods in Cell Biology*, eds. Stein, G., Stein, J. & Kleinsmith, L. J. (Academic, New York), Vol. 16, pp. 125–139.
17. Lane, C. D. & Knowland, J. (1975) in *The Biochemistry of Animal Development*, ed. Weber, R. (Academic, New York), Vol. 3, pp. 145–181.
18. Lane, C. D. (1983) in *Current Topics in Developmental Biology*, eds. Moscona, A. A. & Monroy, A. (Academic, New York), Vol. 18, pp. 89–117.
19. Schrier, B. K., Wilson, S. H. & Nirenberg, M. (1974) *Methods Enzymol.* **32**, 774–777.
20. Kessler, S. W. (1975) *J. Immunol.* **115**, 1617–1624.
21. Pelham, H. R. B. & Jackson, R. J. (1976) *Eur. J. Biochem.* **67**, 247–256.
22. Constantini, F., Britten, R. J. & Davidson, E. H. (1980) *Nature (London)* **287**, 111–117.
23. Anderson, D. M., Richter, J. D., Chamberlin, M. E., Price, D. H., Britten, R. J., Smith, L. D. & Davidson, E. H. (1982) *J. Mol. Biol.* **155**, 281–309.