Collagen Synthesis in Xenopus Oocytes after Injection of Nuclear RNA of Frog Embryos

Abstract. *A messenger RNA fraction from polysomes of frog larvae or RNA preparations from isolated nuclei of developing frog embryos were injected into growing* Xenopus laevis *oocytes that were incubated with labeled proline. Column chromatography of protein hydrolyzates revealed labeled hydroxyproline after injection of the messenger RNA fraction and neurula nuclear RNA, indicating that the injected material had promoted collagen synthesis.*

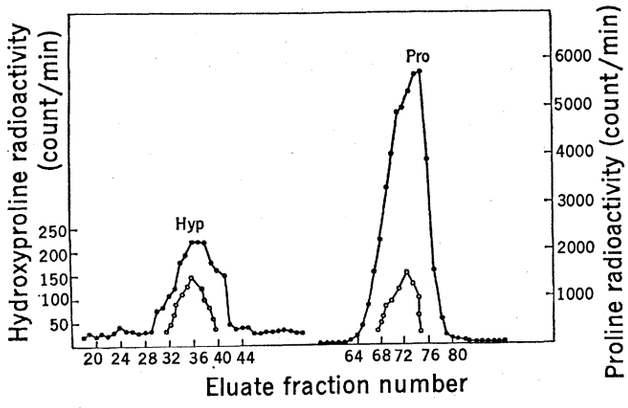
Injection of growing oocytes of *Xenopus laevis* with messenger RNA (mRNA) from mouse reticulocytes results in hemoglobin synthesis (*1*). In the present study, mRNA preparations from frog (*Rana pipiens*) larvae and RNA prepared from isolated nuclei of developing frog embryos were injected into growing *Xenopus* oocytes that were incubated with labeled proline to assay for collagen synthesis. Detection of labeled hydroxyproline in the polypeptides indicates collagen synthesis, because this amino acid is restricted to collagen or elastin (*2*) and no elastin is present in frog embryos or larvae. Collagen synthesis commences at gastrulation, and the rate increases until the larval stage is reached in developing frog embryos (*3*).

Messenger RNA was prepared by releasing ribonucleoprotein from polysomes with ethylenediaminetetraacetic acid (EDTA) and treatment with sodium dodecyl sulfate (*1*). Frog larvae at stage 25 (*4*) were washed in solution A [0.035*M* tris(hydroxymethyl)aminomethane (tris) at *p*H 7.4, 0.015*M* KCl, and 0.1*M* $MgCl_2$] and homogenized in solution A containing polyvinyl sulfate (PVS), 4 μg/ml. The homogenate was centrifuged at 12,000*g* for 15 minutes and the resulting supernatant was layered over 30 percent sucrose (free of ribonuclease) in solution A with PVS and centrifuged at 50,000*g* for 2 hours to sediment polysomes. The polysomes were then suspended in 0.033*M* EDTA. The RNA was separated in a linear sucrose gradient (15 to 30 percent) at 25,000 rev/min in an SW 25.1 rotor (Beckman) for 40 hours. The mRNA fractions, located between 18*S* and 4*S*, were pooled, and NaCl and sodium dodecyl sulfate were added (final concentrations, 0.4*M* and 1 percent, respectively). Two volumes of ethanol were added and the RNA and protein were allowed to precipitate overnight at −20°C. The precipitate was dissolved in 1 percent sodium dodecyl sulfate (1 ml per 100 μg of RNA) and layered over an 8 to 20 percent sucrose gradient and centrifuged for 40 hours. The mRNA fractions were pooled and precipitated in ethanol. The precipitate was dissolved in 0.35*M* tris (*p*H 7.4) and dialyzed overnight against twice-distilled water; the RNA solution was then lyophilized.

Nuclei were isolated in a solution of 0.25*M* sucrose and 2 m*M* $MgCl_2$ (*5*), and washed twice at 600*g* in 0.025*M* citric acid to dissolve yolk platelets. Then RNA was prepared (*6*) from the isolated nuclei. Both the mRNA and nuclear RNA preparations were dissolved in a solution of 0.088*M* NaCl, 0.001*M* KCl, and 0.015*M* tris-HCl (*p*H 7.6).

Oocytes were obtained from *Xenopus* females in which ovulation had been induced by hormone (Antuitrin S, Parke-Davis) 2 to 4 weeks before use (*1*). Growing oocytes (approximate diameter, 1.8 mm) were dissected free of connective tissue and incubated in medium containing [3,4-^{3}H]proline (20 μc/ml) for 2 hours before injection. Each oocyte was then injected with 50 to 70 nl of the mRNA or nuclear RNA (800 μg/ml), by means of hand-drawn micropipettes with a microinjection apparatus (Sensaur) consisting of a syringe and micrometer. The injected oocytes were then returned to the labeled culture medium and incubated for 10 hours at 20°C. The same volume of solution without dissolved RNA was injected in control experiments.

After incubation the oocytes were washed three times in 0.4 percent saline and homogenized in 5 percent trichloroacetic acid. The samples were exhaustively dialyzed against distilled water and then the proteins were hydrolyzed in 6*N* HCl at 120°C for 15 hours. Column chromatography (*7*) was employed to estimate collagen synthesis (*3*). Unlabeled proline and hydroxyproline (100 μg/ml) were added to the samples, and the two amino acids were separated by passage through a 150-cm column containing MS fraction D, Aminex (Bio-Rad) (*3*). The amino acids were eluted in citrate buffer (*p*H 3.25), and the fractions were counted



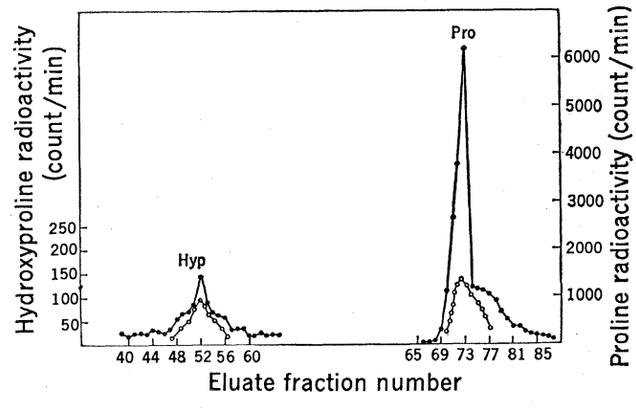


Fig. 1 (left). Elution pattern of labeled hydroxyproline (*Hyp*) and proline (*Pro*), given by solid circles. The absorbance of the added authentic hydroxyproline and proline is indicated by open circles. Fifty-five growing oocytes were incubated for 2 hours in medium containing [^{3}H]proline (20 μc/ml) and then injected with a larval mRNA fraction (intracellular concentration, 15 μg/ml) and returned to the labeled incubation medium for 10 hours. Fig. 2 (right). Elution pattern of labeled hydroxyproline (*Hyp*) and proline (*Pro*) from oocytes injected with neurula nuclear RNA. Fifty-five oocytes were injected with RNA (intracellular concentration, 20 μg/ml) and handled as described for Fig. 1. Solid circles, radioactivity; open circles, absorbance of authentic hydroxyproline and proline.

in a liquid scintillation spectrometer. Radioactivity was summed for each of the two amino acid peaks. The hydroxyproline radioactivity was corrected by a factor of 1.3 because 25 percent of the label of [3,4-^{3}H]proline is lost during conversion (*8*).

The mRNA fraction from *Rana pipiens* induced collagen synthesis in injected oocytes, as shown by the presence of labeled hydroxyproline in the protein hydrolyzate (Fig. 1). The ratio of corrected radioactivity in hydroxyproline to radioactivity in proline was 0.041. Injection of nuclear RNA of early neurulae (stage 14) also induced collagen synthesis in the oocytes, as revealed by the peak of labeled hydroxyproline (Fig. 2); in this case the incorporation ratio was 0.027. Injection of saline or nuclear RNA of early gastrulae (stage 10) (*4*) or of swimming larvae (stage 25) did not promote collagen synthesis, as shown by the absence of a radioactivity peak for hydroxyproline.

The elution patterns of labeled hydroxyproline varied somewhat, probably because of changes in degree of packing of the column. However, the labeled peaks were always identical with the optical density peaks of the added authentic proline and hydroxyproline. When the column fractions containing labeled hydroxyproline and proline were rechromatographed with authentic hydroxyproline and proline, the optical density and radioactive peaks coincided exactly. All experiments and control series were repeated three or four times with similar results.

Our most important result is that neurula nuclei contain RNA that promotes collagen synthesis in injected *Xenopus* oocytes, whereas activity of injected larval nuclear RNA cannot be detected. The absence of collagen synthesis after injection of larval nuclear RNA probably is not caused by a complete absence of the RNA that promotes collagen synthesis, but rather by the lower quantity of this RNA. This appears surprising because larvae synthesize much more collagen than do early neurulae (*3*). This synthesis can be explained by the greater conservation of collagen mRNA in the cytoplasm at the later developmental stage (*6*), even though transcription is restricted in the nuclei (*9*). Nuclear RNA from early gastrulae was inactive in promoting collagen synthesis, because synthesis of DNA-like RNA is just commencing again at this stage after being absent during cleavage (*10*).

J. W. Rollins*
R. A. Flickinger
Department of Biology, State University of New York, Buffalo 14214

References and Notes

1. J. B. Gurdon, C. S. Lane, H. R. Woodland, G. Marbaix, *Nature* **233**, 177 (1971); C. D. Lane, G. Marbaix, J. B. Gurdon, *J. Mol. Biol.* **61**, 73 (1971); V. A. Moar, J. B. Gurdon, C. D. Lane, G. Marbaix, *ibid.*, p. 61.
2. M. Mathews, *Biol. Rev. Cambridge Phil. Soc.* **42**, 499 (1967).
3. H. Green, B. Goldberg, M. Schwartz, D. Brown, *Develop. Biol.* **18**, 391 (1968); J. Klose and R. A. Flickinger, *Biochim. Biophys. Acta* **232**, 207 (1971).
4. W. Shumway, *Anat. Rec.* **78**, 139 (1940).
5. C. F. Graham, K. Arms, J. B. Gurdon, *Develop. Biol.* **14**, 349 (1966).
6. R. F. Greene and R. A. Flickinger, *Biochim. Biophys. Acta* **217**, 447 (1970).
7. S. Moore, H. Spackman, W. H. Stein, *Anal. Chem.* **30**, 1185 (1958).
8. D. J. Prockop, P. S. Ebert, B. M. Shapiro, *Arch. Biochem. Biophys.* **106**, 112 (1964).
9. W. C. Claycomb and C. A. Villee, *Exp. Cell Res.* **69**, 430 (1971).
10. D. Brown and E. Littna, *J. Mol. Biol.* **20**, 81 (1966).
11. Research supported by NIH grant GM 16236-03.
* Present address: Department of Pathology, New York University Medical School, New York 10016.

26 May 1972; revised 13 October 1972

Circadian Corticosteroid Periodicity: Critical Period for Abolition by Neonatal Injection of Corticosteroid

Abstract. *Circadian variation of corticosteroid concentrations in rat plasma is suppressed if corticosteroids are administered between days 2 to 4 of neonatal life, but not if they are given between days 12 to 14 of neonatal life. This indicates a critical period for the effect of corticosteroid administration on the central nervous system pathways regulating such periodicity. Circadian periodicity of corticosteriods is not affected by neonatal administration of testosterone or reserpine.*

Circadian periodicity of plasma corticosteroid concentration is absent at birth, and gradually appears as the organism matures (*1*). In the rat, such periodicity becomes established 21 to 25 days after birth, a time much later than the appearance of the capacity of the hypothalamic-pituitary-adrenal axis to respond to stress and steroid feedback (*2*). The appearance of cyclicity of luteinizing hormone release in the adult female rat is dependent on the concentration and nature of gonadal steroid present immediately after birth (*3*), and is also affected by neonatal administration of drugs (such as reserpine and chlorpromazine) presumed to affect neurotransmitter content in the central nervous system (*4*). The data in the present report indicate that the circadian periodicity of plasma corticosteroid concentration can also be suppressed following the administration of corticosteroids (but not reserpine) during a critical period after birth.

Newborn Sprague-Dawley rats were identified as to litter source and housed in a light-regulated, temperature-controlled room. Animals were weaned and separated according to sex at 21 days of age, and were handled daily from the time of weaning. Experimental groups consisted of controls and animals injected subcutaneously with equal volumes of saline, corticosterone, testosterone, hydrocortisone, or dexamethasone. The drug dosages employed and the ages of animals at the time of injection are indicated in Fig. 1. For each category, groups of six animals (usually three of each sex), 30 days of age, were decapitated at 4-hour intervals during a 24-hour period, and trunk blood was collected for plasma corticosteroid determination (*5*). Assays were performed in duplicate on samples from individual animals. The animals chosen for each sampling time represented a cross section of the litters available in a particular study. Data from this laboratory (*6*) and elsewhere (*7*) indicated plasma corticosteroid concentrations and patterns are similar in male and female animals of this age, so that results for both sexes were combined.

Dexamethasone or hydrocortisone, given 2 to 4 days after birth, virtually suppressed the circadian periodicity of plasma corticosteroid concentrations at 30 days of age; hydrocortisone had the greater effect. (The mean extent of circadian variation of plasma corticosteroids was 8.2 μg/100 ml in the group receiving dexamethasone, 2.7 μg/100 ml in the hydrocortisone-treated group, 22.8 μg/100 ml in controls, and 21.7 μg/100 ml in those receiving saline.) A similar dose of dexamethasone administered 12 to 14 days after birth had no effect on circadian variation. Corticosterone, testosterone, or reserpine, given 2 to 4 days after birth, was also