PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **147,** 318–322 (1974)

Poly(A)-Attached RNA as Activator in Embryonic Differentiation (38334)

M. C. NIU, A. K. DESHPANDE, AND L. C. NIU

Department of Biology, Temple University, Philadelphia, Pennsylvania 19122

Nuclear transplantation experiments have shown that cytoplasm of the fertilized eggs can control certain aspects of nuclear activity (1). Poly (A)-attached RNA (mRNA) is one of the most active cytoplasmic components and capable of initiating specific differentiation of chick blastoderm (2), mouse ascites cells (3, 4), mouse uterus (5, 6), and chick oviduct (7). Autoradiographic study on the distribution of exogenous [^{3}H]RNA in mouse ascites cells revealed more silver grains localized in nucleus than in cytoplasm (8). These RNA-treated cells had increased synthesis not only of RNA (9) but also of specific protein (3–7). The RNA-stimulated activity was sensitive to actinomycin D (9). In cell-free system, only nuclear RNA of the naturally occurring RNAs was capable of promoting RNA synthesis in thymocyte chromatin (10). Biosynthesis of the chromatin from sweetpea was stimulated by chromosomal RNA (11). All these data indicate that exogenous RNA plays an important role in the regulation of nuclear (DNA) function. However, mRNA has lately been proposed to "transform" gene function. This is based on the newly discovered enzyme, reverse transcriptase, in chick embryo (12). According to this proposal, mRNA and the enzyme prime the synthesis of new DNA. It would soon be incorporated into the genome and thus responsible for the synthesis of new species of mRNA. The translation product of the new mRNA, protein or enzyme, would be the type of mRNA donor tissue. On the contrary, the activation or derepressor theory demands that both transcription and translation products, mRNA and protein, respectively, are of the types of the recipient cells.

The authors have set to test experimentally the alternate theories by the use of a system called "embryonic induction." Undifferentiated tissue, *e.g.*, the postnodal piece (PNP) of the stage 4 chick blastoderm, is incapable of differentiating into tissue other than epithelial cells, red blood cells, and mesenchyme. If it was treated with chicken heart RNA, the explants developed beating tube and/or tissue (2). The question asked is: will calf heart mRNA be able to initiate the differentiation of PNP into beating tube and/or tissue? The aim of this communication is to show that calf heart mRNA functions almost as well as chicken heart mRNA in inducing heart formation. As to the type of heart formed (chick or calf), we have used the pattern of lactate dehydrogenase in chicken and calf hearts as criterion for identification. The isozyme was analyzed by gel electrophoresis. The results showed that both chicken and calf heart mRNA-induced beating tissues had the same pattern of lactate dehydrogenase typical of the chicken heart.

Materials and Methods. Fertilized eggs of White Leghorn hens were purchased from Shaw's Hatchery, West Chester, PA, and incubated at 38° to get the definitive primitive streak stage (stage 4, Hamburger and Hamilton (13)). The blastoderms were explanted, and the area opaque was trimmed off. The postnodal piece (PNP) was obtained by transecting the area pellucida 0.6 mm behind the Hensen's node. The explant thus obtained contained no presumptive heart-forming area (14, 15). The method of cultivation is modified after New (16) and Chauhan and Rao (17). The PNP was transferred to the yolk-free vitelline membrane (VM) previously mounted around the glass ring, with hypoblast facing up. The excess Pannett-Compton solution (PC) was removed. One milliliter of the nutritive medium was added to the watch glass. The nutritive medium used was a whole-egg extract prepared by thoroughly mixing whole egg with 50 ml Ringer solution. The homogenate was centrifuged at 2000 rpm for 30

min at 4°. The supernatant was mixed with an equal volume of PC solution. The pH of the medium was 7.6–7.7.

Preparation of poly (A)-attached RNA (mRNA). Both calf and chicken hearts in groups of 70 g were used for the preparation of mRNA and filtrate RNA. The procedure of isolation and purity tests have already been described elsewhere (20).

RNA-treatment. To the PNP explant prepared earlier, 0.1 ml of PC solution with and without RNA (80 OD/ml) was applied inside and outside the VM. The RNAs used were chicken heart mRNA, chicken heart filtrate RNA, and calf heart mRNA. All cultures were incubated at 37.5°. The medium was changed every 24 hr with fresh egg extract medium. Observation was made daily. On Day 5 (120 hr) of cultivation, some of them were fixed in Bouin's fluid and examined histologically and the rest were collected in saline and frozen quickly in dry ice–acetone mixture. They were saved for analysis of the isozyme, lactate dehydrogenase (LDH) using the procedure of Markert (21). For identification purposes, the LDH patterns of normal chick heart, adult chicken heart, and calf heart were studied and compared with the enzyme from hearts induced by chicken and calf heart mRNAs.

The effect of inhibitors. Both actinomycin D and bromodeoxyuridine were purchased from Nutritional Biochemicals. The concentration of the former used was 0.2 μg per explant and the latter was 3 μg. They were used separately from mRNA and simultaneously. The concentration of ribonuclease used was 50 μg in 40 OD/ml.

High-resolution autoradiography. Chicken heart mRNA was methylated with [^{3}H]dimethyl sulfate (22). The [^{3}H]RNA thus obtained was used to treat PNP for 1 hr in room temperature. The explants were washed with saline and then fixed in glutaraldehyde for 2 hr on ice. After washing three times with glutaraldehyde buffer (pH 7.4) they were refixed individually in 2% osmium tetroxide for 1 hr on ice. Embedding was made in Araldite. An automatic Blum–Porter microtome was used to cut 0.5-μm and 90–150-nm sections. Gold-colored sections were chosen and mounted on grids. The method for electron microscopic autoradiography was published elsewhere (8).

Results. High-resolution autoradiography. By electron microscopy, two types of cells were found in PNP: one is abundant and electron light and the other rare and electron dense. In the autoradiographs we have examined, silver grains were seen mostly in electron-light cells. A total of 500 PNP cells with silver grains were counted. The distribution was 2836 grains in nuclei and 1897 in cytoplasm. The ratio of silver grains between nuclei and cytoplasm is 1.5. In other words, more grains are in nucleus than in cytoplasm.

Heart-forming capacity of heart mRNA. A. *From chicken.* I. *Control series.* The primitive streak of the explants disappeared after 24 hr of incubation. The explants rounded up in 48 hr. Red patches of blood cells were seen on the third day. However, twitching tissue has never been observed.

II. *Experimental series.* The explants of these series appeared more compact than the control at the end of 24 hr of incubation. They usually took up shapes of various kinds. Red patches developed in the vesicular explants on the third day and twitching area(s) on the next day. The twitching evolved to rhythmic beating on the fifth day. The beating rate averaged 20–25 per min.

1. *mRNA.* Thirty explants were treated with mRNA. Practically all differentiated (96%). Twenty-seven of the 30 explants (90%) developed beating tube and tissue. The rhythmic pulsation averaged 22 per min. Neural and tubule formation occurred at the frequency of 60% and 40%, respectively.

(a) The effect of mRNA concentration. Seven concentrations of mRNA were used to treat 129 PNPs (80, 60, 40, 20, 10, 5, and 1/4 OD per ml). First beating was found on Day 4. The rate averaged 20 per min on Day 5. Examination of serial sections of all 129 explants revealed the presence of a variety of tissues: beating tube, beating tissue, neuroid, notochord, somite, and tubule. Their distribution is summarized in Table I. It can be seen that the frequency of heart formation is directly proportional to the concentration used (Fig. 1). The concentration does not affect the general frequency of differentiation (column 3, Table I), nor the frequency of other organ formation (last 4 columns, Table I).

(b) The effect of ribonuclease. Twenty-five PNPs were treated with RNase-digested mRNA. One of them developed beating tissue. In contrast, the corresponding concentration of intact

TABLE 1. The Effect of Heart mRNA on the Development of Excised Chick Blastoderm.

Concn used (OD/ml)	No. of PNPs	% of Dif'n	No. and % (in parentheses) of development of various organs					
			Beating heart	Beats/min.	Neuroid	Notochord	Somite	Tubule
80	30	96	27 (90)	22	18(60)	-(—)	2(8)	12(40)
60	13	100	10 (77)	22	6(46)	2(15)	3(23)	7(54)
40	24	92	18 (75)	21	7(29)	2(8)	1(4)	8(33)
20	24	88	15 (63)	15	17(71)	8(33)	1(4)	10(42)
10	14	91	6 (43)	22	11(79)	-(—)	2(14)	1(7)
5	13	91	4 (31)	11	7(54)	-(—)	1(8)	4(31)
1/4	11	81	2 (18)	18	5(45)	-(—)	1(9)	4(36)
Filtrate[a]-80	28	81	3 (11)	20	9(32)	2(8)	3(13)	10(36)
0(controls)	48	6	— (—)	—	3(6)	2(4)	-(—)	—(—)

[a] This is the fraction of whole heart RNA that passes through Millipore filter.

mRNA (40 OD per ml) initiated heart formation in 75% of the explants. The loose type of cardiac tissue was found in three of the 25 explants. The differentiation of the other tissue was not affected by RNase treatment. It appears that mRNA possesses both specific and nonspecific function. While specific function was sensitive to RNase, the nonspecific function was indifferent to RNase.

2. *Filtrate RNA*. This is the fraction of heart RNA with mRNA selectively removed. Of 28 treated explants, three developed beating tissue (11%). The frequency of other tissue formation was similar to that of heart mRNA-treated series. The similarity in nonspecific organ formation between filtrate and mRNA series reveals a point of great interest, namely, of the cellular RNAs, only mRNA is able to induce specific organ (heart) formation. The low percentage of beating tissue in the filtrate series is likely due to mRNA contamination.

B. From calf. The inducing capacity of calf heart mRNA was compared with chicken heart mRNA. A total of 55 explants were cultured and 45 developed pulsating tube and/or tissue (82%). The heart-forming frequency between chicken and calf heart mRNAs was analyzed statistically. The difference was insignificant.

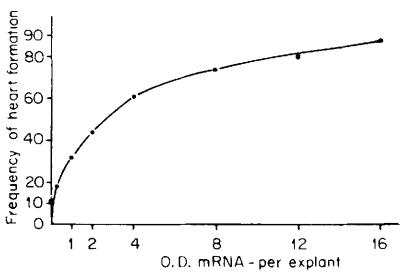

FIG. 1. A graph showing the concentration effect of heart mRNA on the development of beating hearts in explanted PNPs.

Effect of inhibitors on heart mRNA. A. Actinomycin D. Actinomycin D is known to bind with guanine residues of DNA and thus inhibit its participation in transcription. The effect of actinomycin D on the differentiation of heart mRNA-treated PNP was studied under two experimental conditions:

1. *Preincubation*. Twenty-five PNPs were incubated with actinomycin D (0.2 μg per explant) for 2 hr in room temperature. They were washed once with PC solution and then treated with mRNA and cultured as usual. None of them developed beating tissue.

2. *Simultaneous incubation*. Twenty-four PNPs were treated with actinomycin D and mRNA simultaneously. They were cultured at 37.5° as usual. Three of the 24 explants developed beating tissue.

B. Bromodeoxyuridine. Twenty-five PNPs received simultaneous treatment with bromodeoxyuridine and mRNA. Four of them developed beating tissue.

Gel electrophoresis. In order to answer the question concerning the species property of the calf heart mRNA-induced beating tube and/or tissue, the pattern of isozyme (lactate dehydrogenase) was chosen as the criterion distinguishing the calf heart mRNA-induced hearts from calf heart. This was accomplished by gel electrophoresis using the following tissue homogenates: (1) Stage 4 chick blastoderm; (2) chick and chicken hearts; (3) chicken heart mRNA-induced hearts; (4) calf heart mRNA-induced

hearts; (5) calf heart.

The results were clear-cut (Fig. 2). Series 1–4 had one band which is equivalent to isozyme IV of lactate dehydrogenase. In contrast, series 5 had four isozymes which are equivalent to isozymes I, II, III, and IV. Therefore, the calf heart mRNA-induced hearts possess lactate dehydrogenase typical of chicken heart.

Discussion. Whole heart RNA was filtered through nitrocellulose membrane (Millipore filter). The filtrate contained ribosomal and transfer RNAs while the eluate from the membrane had the poly (A)-attached RNA (mRNA). The frequency of heart formation, *i.e.*, beating tube (20) and/or tissue (18, 19), in the filtrate (11%) and eluate (88%) indicates that the heart-forming component of the heart RNA is mRNA. The development of beating tissue in the filtrate series was likely due to the mRNA contamination. The frequency of heart formation varied with the concentration of mRNA used. The concentration had no effect on other tissue formation, *e.g.*, neural tissue, notochord, somite, or tubule. These latter tissues could also be induced by the unspecific action of the filtrate.

The heart-forming capacity of heart mRNA was specific because mRNA isolated by the same procedure from brain (20) and kidney (unpublished) as heart mRNA failed to induce heart formation. Furthermore, pancreatic ribonuclease was found to significantly reduce the heart-forming capacity of heart mRNA.

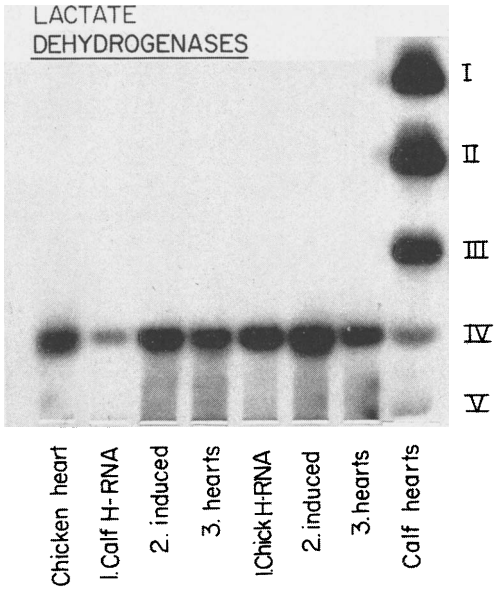

FIG. 2. The distribution of LDH activity among the adult chicken heart; the calf heart mRNA-induced hearts (1–3); the chicken heart mRNA-induced hearts (1–3); and the adult calf heart. Note the presence of isozyme IV in the calf heart mRNA-induced hearts.

The ^{3}H-labeled heart RNA was traced in cells of PNP by high-resolution autoradiography. Silver grains appeared more in nucleus than in cytoplasm. The preferred nuclear localization of mRNA fits well with the fact that the effect of mRNA was sensitive to actinomycin D and bromodeoxyuridine. The latter effect shows that exogenous RNA in cells of PNP is engaged in the regulation of nuclear activity. Thus, when PNP explants were treated with chicken and calf heart mRNA, 90% and 82% of them developed beating tube and/or tissue, respectively. The difference in frequency between chicken and calf heart mRNA is statistically insignificant.

The mode of the RNA action has lately been the subject of intensive study. Active workers in the field have provided evidence showing that mRNA plays a role in transcription (10–12). Should calf heart mRNA activate the genome, the target tissue, PNP, would have chicken heart developed. A classical example of this kind is shown by the reciprocal transplantation of the head ectoderm between frog and newt's neurulae (23). This head ectoderm differentiates into epidermis if grafted onto other part of the embryo. When it is transplanted in front of the head without disturbing the underlaying mesenchyme (inductor tissue), frog ectoderm on newt's head develops sucker instead of host's balancer and, conversely, newt ectoderm on frog's head develops balancer instead of host's sucker. If, on the other hand, calf heart mRNA "transforms" PNP into beating tissue, the induced heart would have to be of calf type. An example of this kind is the transformation of normal cells into cancerous tissue by RNA from oncogenic RNA virus (24).

The isozymes of lactate dehydrogenase (LDH) are present in all kinds of somatic tissues. Isozyme IV only was found in stage 4 chick blastoderm, embryonic and adult chicken hearts (25). In contrast, IV and V were found in fetus heart of cow (unpublished) and I, II, III, and IV in calf heart. The difference of isozymes between chick and cow's fetus heart has been taken as a diagnostic tool in determining the species to which the mRNA-induced heart does belong. In view of the fact that isozyme IV was found only in the calf heart mRNA-induced heart, we are

inclined to believe that calf heart mRNA can regulate but can not transform the genome of the undifferentiated cells (26).

Summary. Poly(A)-attached RNA (mRNA) was isolated from chicken and calf hearts. Both mRNAs were found to induce the development of explanted postnodal pieces (PNP) of stage 4 chick blastoderm into beating tubes and/or tissues. Heart formation is specific and the frequency was found to depend entirely upon the concentration of mRNA used. In contrast, the concentration had no influence on other tissue formation *e.g.*, neural tube, tubules, myoblasts, and/or notochord.

The uptake of heart [^{3}H]RNA by PNP cells was analyzed using high-resolution autoradiography. Silver grains were localized more in nucleus than in cytoplasm. The effect of heart mRNA on PNP was sensitive to actinomycin D and bromodeoxyuridine. It appears, therefore, that exogenous mRNA plays an important role in nuclear function. This RNA may affect the nuclei of intact cells in two ways: (1) as a derepressor, and (2) as a primer of new DNA synthesis. These alternatives were tested experimentally using the isozyme, lactate dehydrogenase, as marker. Homogenates of the chicken and calf heart mRNA-induced beating tissues were subjected to gel electrophoresis. Both gels had one band (isozyme IV) that was typical of the chicken heart. Accordingly, the mRNA activates but does not transform the genome of embryonic chick tissue.

This study was supported in part by research grants from The Population Council and The National Foundation. The authors thank Professor Clement Markert for showing one of us (M. C. N.) the technique of his starch gel electrophoresis and for confirming the pattern of lactate dehydrogenase shown in Fig. 2.

1. Gurdon, J. B., *in* "Problems in Biology: RNA in Development" (E. W. Hanly, ed.), p. 217. University of Utah Press, Salt Lake City (1969).

2. Niu, M. C., and Deshpande, A. K., J. Embryol. Exp. Morphol. **29,** 485 (1973).

3. Niu, M. C., Cordova, C. C., and Niu, L. C., Proc. Nat. Acad. Sci. USA **47,** 1689 (1961).

4. Granzow, C. J., Niu, L. C., Lin, T., and Niu, M. C., Amer. Zool. **11,** 684 (1971).

5. Mansour, A. M., and Niu, M. C., Proc. Nat. Acad. Sci. **53,** 764 (1965).

6. Niu, M. C., Niu, L. C., and Yang, S. F., *in* "The Role of RNA in Reproduction and Development" (M. C. Niu and S. Segal, eds.), p. 90. Elsevier, New York (1973).

7. Tuohimaa, P., Segal S. J., and Koide, S. S., Proc. Nat. Acad. Sci. USA **69,** 2814 (1972).

8. Niu, M. C., Niu, L. C., and Guha, A., Proc. Soc. Exp. Biol. Med. **128,** 550 (1968).

9. Niu, M. C., Lin, T., and Niu, L. C., Proc. Soc. Exp. Biol. Med. **140,** 256 (1972).

10. Frenster, J. H., Nature (London) **206,** 1269 (1965).

11. Elgin, S. E., and Bonner, J., *in* "The Biochemistry of Gene Expression in Higher Organisms" (J. W. Lee and J. K. Pollak, eds.), Reidel, Dordrecht, Holland (1972).

12. Kang, C. Y., and Temin, H. M., Proc. Nat. Acad. Sci. USA **69,** 1550 (1972).

13. Hamburger, V., and Hamilton, H. L., J. Morphol. **88,** 49 (1951).

14. Rudnick, D., Anat. Rec. **70,** 351 (1938).

15. Mulherkar, L., J. Embryol. Exp. Morphol. **6,** 1 (1958).

16. New, D. A. T., J. Embryol. Exp. Morphol. **3,** 326 (1955).

17. Chauhan, S. P. S., and Rao, K. V., J. Embryol. Exp. Morphol. **32,** 71 (1970).

18. DeHaan, R. L., Carnegie Inst. Year Book **70,** 66–84. (1970–1971).

19. Rawles, M. E., Physiol. Zool. **15,** 22 (1943).

20. Deshpande, A. K., Niu, L. C., and Niu, M. C., "The Role of RNA in Reproduction and Development," p. 229. Elsevier, New York (1971).

21. Markert, C. L., and Utsprung, II., Develop. Biol. **5,** 363 (1962).

22. Smith, K. D., Armstrong, J. L., and McCarthy, B. J. Biochim. Biophys. Acta **142,** 323 (1967).

23. Spemann, H., "Embryonic Development and Induction." Yale Univ. Press, New Haven (1938).

24. Temin, H. M., Proc. Nat. Acad. Sci. USA **69,** 1016 (1972).

25. Lindsay, D. T., J. Exp. Zool. **152,** 75 (1963).

26. Niu, M. C., *in* "Axenic Mammalian Cell Reaction" (G. Tritsch, ed.), p. 155. Marcel Dekker, New York (1969).

Received Feb. 14, 1974. P.S.E.B.M., 1974, Vol. 147.