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Embryonic gene regulation: role of an inducer RNA in manipulation of embryonic gene functions

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Regulación de genes embrionarios: rol de un RNA inductor en la manipulación de las funciones de genes embrionarios

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The problem of the origin of structure in development is an old one. Early embryologists were faced with the seemingly impossible task of formulation of a conceptual framework which will provide a satisfactory basis for understanding the mechanisms of embryonic development and morphogenesis. According to the current concepts, the fertilized egg contains, in addition to the DNA genome of the nucleus, "informational molecules", possibly diffusible and macromolecular in nature, which are regionalized assymetrically in the cytoplasm (1, 2). These molecules play a key role in determining the early pattern of morphogenetic changes. Morphogenesis and cell differentiation in turn are the results of derepression of specific sets of genes. The "informational molecules" must, therefore, be involved in some way in control of the transcriptional activity of the genome. Models have been proposed in which both RNA and proteins are implicated as regulatory elements affecting the pattern of gene activity (1-5). Although, to our knowledge, there is no direct and clear evidence in support of the main theme of the model, there is ample documentation on manipulation of gene expression in general mediated by both RNA and protein. The role of non-histone chromosomal protein(s) in regulation of transcription of the stage specific reconstituted chromatin has been well documented (see Stein and Stein, this issue (6) and refs. therein). A particularly relevant example is that of a protein in Mexican axolotl synthesized during oogenesis which seems to be essential for activation of nuclear genes required during gastrulation and organogenesis (7). The involvement of RNA has been postulated in control of several cellular functions, such as in specification of antibody production (8), interferon induction (9), in causing specific disease symptoms in plant tissues (10), and in others where RNA are implicated in control of cell development and differentiation (see refs. 5, 11, 12, 13 for reviews). In spite of these intriguing examples, there is no clear definition of the physiological role played by these RNA and protein molecules. Experimental systems, therefore, which permit the identification and the functional analysis of potential regulatory elements are highly suitable for elucidation of mechanism(s) underlying cell development and differentiation.

In the stage 4 chick blastoderm, an area located 0.5 mm posterior to Hensen's node, the

postnodal piece (PNP), consists of undifferentiated population of cells, since the explants when cultivated *in vitro* in a variety of media or by choriollantoic grafting technique do not develop into any histologically identifiable structures (14-17). However, the anterior portion of the blastoderm including Hensen's node (AP) alone can develop into structures, such as the embryonic heart tube similar to the intact embryo (Fig. 1) suggesting that the developmental potentiality for at least the heart formation is localized in the AP at this stage of development (see ref. 20). Later studies (17-19) have reported that the PNP can be induced to develop into specific tissues, such as the heart muscle tissues, when the explants are cultured in the presence of RNA isolated from differentiated chick embryonic heart. The development appears to be organ specific since RNAs from kidney or thymus are incapable of inducing heart formation. The difficulty, however, in interpreting this novel mode of development is the lack of information on identification of the RNA involved and of adequate criteria for characterizing the differentiative transition.

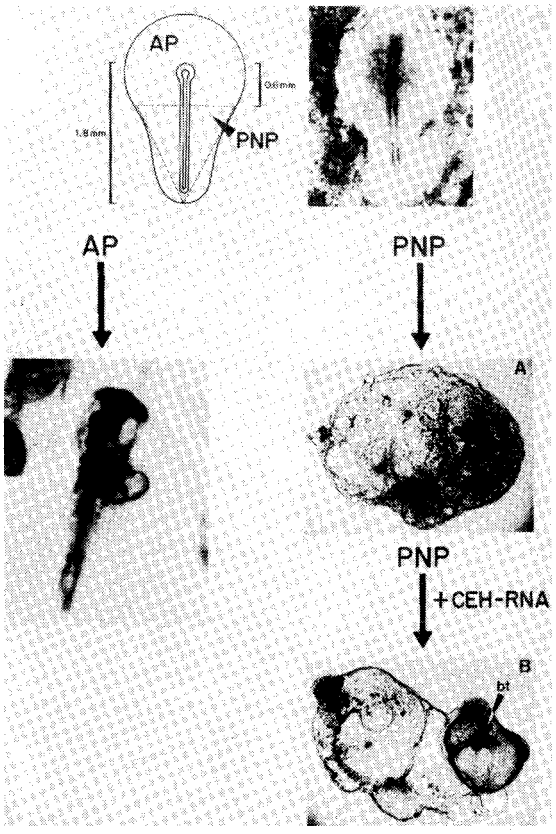


Fig. 1. Stage 4 chick blastoderm characterized by a fully developed primitive streak (1.8 mm) is obtained after incubation of fertile chicken eggs for 20-22 hr at 38°C as described earlier (20). The explants, the post-nodal piece (PNP) and the anterior pierce (AP) are obtained by transection of the blastoderm at 0.6 mm posterior to Hensen's node. AP when cultivated *in vitro* (18) develops into embryonic structures including a well-defined heart tube, whereas the PNP under identical conditions fails to develop into any histologically identifiable structures (A). Addition of the inducer RNA obtained from the 16-day old chick embryonic heart (see text and ref. 20) to the PNP culture promotes the formation of the heart tube (bt) which starts spontaneous and rhythmic pulsations (B). The development appears to be similar to the embryonic cardiogenic process (see text and ref. 20). Addition of the same RNA to the AP causes no apparent change in development.

We, therefore, undertook to re-examine the phenomenon, RNA-dependent induction of heart-specific tissues in the PNP, in an attempt to i) ascertain that the transition is indeed dependent upon an RNA species; ii) to establish an adequate criteria for characterization of the differentiation; iii) to isolate, purify and characterize the RNA competent of inducing the specific changes, and eventually, iv) to investigate whether RNA-induced transformation *in vitro* have any biological significance in development and differentiation *in vivo*. We have recently demonstrated unequivocally (20-22) that the appearance of a specific mode of morphological and biochemical changes in the PNP of stage 4 chick blastoderm is indeed dependent upon the addition of an RNA species obtained from the differentiated chick embryonic heart. The changes that characterize the differentiation in the PNP are similar to those of embryonic cardiogenesis. The inducer RNA is of low molecular weight (7S) and rich in adenylate residues (22). In the absence of the RNA, the PNP remains undifferentiated and a variety of RNAs from other sources isolated under identical conditions and synthetic polynucleotides are not effective in inducing the differentiation.

Isolation, Purification and Characterization of Inducer RNA

Total RNA was obtained by repeated phenol: chloroform: isoamyl-alchohol extration of 16-day old chick embryonic heart homogenate as described previously (20). About 600 A_{260}

units of RNA were routinely obtained from a 200 g batch of 16-day old embryonic hearts. The RNA was then fractionated by oligo (dT) cellulose column chromatography by two-step elution of bound RNA, first with 100 mM NaCl, 10 mM Tris-Cl, pH 7.5, and 0.5% SDS (bound I RNA) and then with 10 mM Tris-Cl, pH 7.5, and 0.5% SDS (bound II RNA) as before (20) (Fig. 2). The RNA from each fraction was recovered by two ethanol precipitations, dialyzed against H_2O and kept frozen at -70°C. When these RNA fractions (unbound, bound I, bound II) were tested for their activity to induce differentiation in the PNP cells (see below), the bound II fraction was the only RNA fraction that possessed the capacity to induce the specific mode of differentiation described below (20-22).

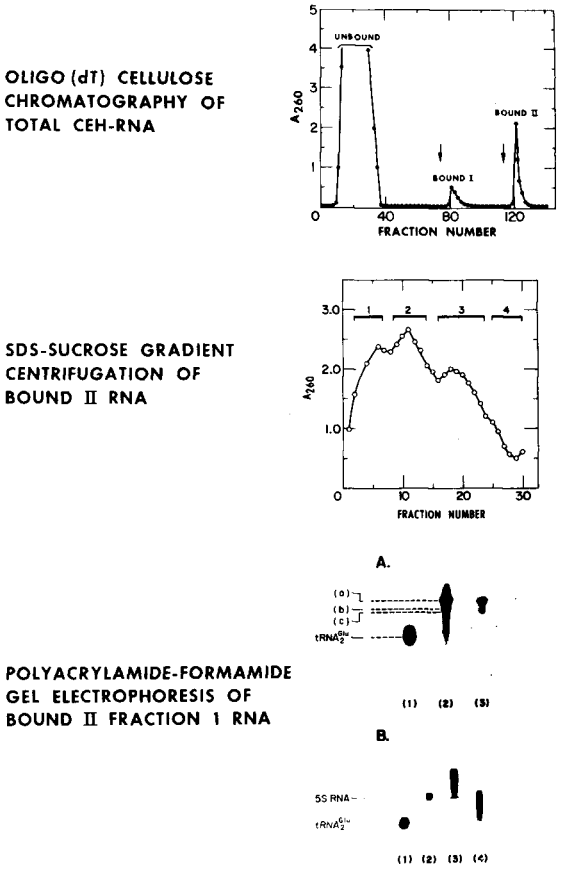


Fig. 2. Purification of heart inducer RNA. Total RNA extracted from the 16-day old chick embryonic heart (22) was fractionated into the unbound, bound I and bound II RNA fractions by oligo (dT) cellulose column chromatography as described earlier (20). The bound II fraction was then centrifuged on 15-30% sucrose density gradient (22). The sedimentation was from left to right. The RNA from fraction 1 was pooled and purified further on a 7.0% polyacrylamide gel in 98% formamide (22) (A) with $tRNA_2^{Glu}$ from *E. coli* as markers (A-1). The resulting three bands, (a), (b), and (c) were recovered from the gel; band (a) was pooled separately from the bands (b) and (c), which were mixed together. Re-electrophoresis of the fractions, (a) and (b) + (c) shows minimal cross contamination (B). *E. coli* $tRNA_2^{Glu}$ (1) and 5S rRNA (2) were used as markers. B(3), Fraction (a); B(4), Fraction (b) + (c).

In an effort to isolate the inducer RNA from the bound II fraction, the bound II RNA was subjected to SDS-sucrose gradient centrifugation (Fig. 2). The gradients consisted of 15-30% sucrose in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA-Na_2, 150 mM NaCl and 0,5% SDS. The centrifugation was done at 25°C at 20,000 rpm for 24 hr in a Beckman SW 27 rotor and fractions of 0.5 ml each were collected from the top using Auto Densi-Flow C fractionator (Buchler Instruments). The RNA from fraction 2 and 3 pooled separately were examined on a 4.2% polyacrylamide gel and the RNA from fraction 1 was run on 7.0% polycrylamide gel containing 98% formamide. Fraction 2 RNA migrated with a mobility of about 18S, whereas fraction 3 contained RNA migrating as 28-29S and some 18S contaminant from fraction 2 (22). Based on the reported sedimentation values for the heavy chain myosin and actin mRNAs (23, 24), it appears that fractions 2 and 3 contained the putative actin and myosin mRNAs respectively. Fraction 1 contained low molecular weight RNA (see below) while fraction 4 (not shown) barely entered the gel. To ascertain whether each of these fractions did indeed contain poly(A), hybridization with [^{3}H]poly(U) was carried out. Fig. 3 shows the saturation hybridization experiment done with varying amounts of [^{3}H]poly(U) and a constant amount of the RNA fractions. All bound II RNA fractions contained hybridizable poly(A) segments. The bound I RNA hybridized little while the unbound RNA did not hybridize. Upon testing the poly(A) RNA fractions for their activity to induce differentiation in the PNP, fraction 1 was found to be most active (Table 1). The fraction 1 RNA was purified on 7.0% polyacrylamide gel in formamide (Fig. 2). The RNA was resolved in at least three bands. RNA from the top band 'a' was eluted separately but the RNA from bands 'b' and 'c' were eluted together. Re-electrophore-

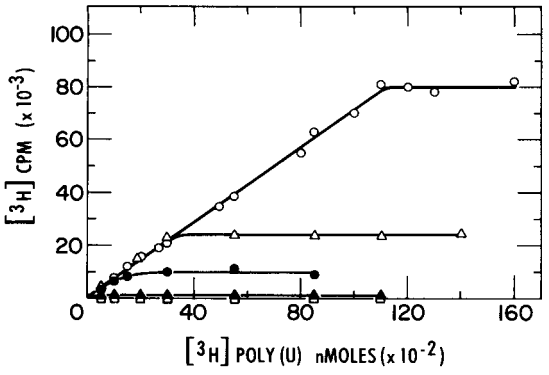


Fig. 3. Saturation hybridization of CEH-RNA fractions with [^{3}H]poly(U). Bound I (▲), Bound II, Fraction 1 (0), Bound II, Fraction 2 (Δ), Bound II, Fraction 3 (●), and unbound RNAs were hybridized with varying amount of [^{3}H]poly(U) as described earlier (22). For further details see ref. 22.

sis of these RNA fractions along with *E. coli* $tRNA_2^{Glu}$ and 5S rRNA, used as markers, shows (Fig. 2) that these two RNA fractions had minimal cross contamination. The trailing effect of RNA in polyacrylamide gel was usually observed when poly(A) containing RNA was run for prolonged periods. Fractions 1'a' and b' + c' RNAs eluted from gel were freed of polyacrylamide particles by rechromatography on oligo(dT) cellulose (not shown) and tested for their activities to induce the changes in PNP (Table 1 and Fig. 4). RNA from bands 'b' and 'c' could no be recovered in an amount sufficient to test its inducing capacity at several concentrations, but a preliminary test showed that at 0.0025 A_{260}/ml this fraction had negligible effect in relation to fraction 1 'a' RNA. The effect of fraction 1 'a' was maximum

TABLE I

RNA-dependent differentiation in PNP

Additions	A_{260}[a]	*N° of PNP Used*	*N] of PNP Differentiated*[b]
Chick embryonic heart RNA:			
Total RNA	15.00	94[c]	60[c]
Unbound RNA	5.00	10	0
Unbound RNA	15.00	51	0
Bound I RNA	0.25	8	0
Bound I RNA	0.50	9	0
Bound II RNA	0.07	9	3
Bound II RNA	0.14	9	5
Bound II RNA	0.28	42[c]	30[c]
Bound II, fraction 1	0.01	19	16
Bound II, fraction 1	0.02	6	5
Bound II, fraction 1	0.05	9	6
Bound II, fraction 2	0.03	6	0
Bound II, fraction 2	0.07	6	0
Bound II, fraction 3	0.02	6	1
Bound II, fraction 3	0.08	8	3
Bound II, fraction 4	0.05	4	9
Bound II, fraction 1 'a'	0.005	20[c]	15[c]
Bound II, fraction 1 'a'	0.01	9	6
Bound, fraction 1 'a' (heat treatment)	0.01	9	6
Bound II, fraction 1 'a' (pan. RNase treated)	0.01	9	0
Bound II, fraction 1 'a' (RNase A treated)	0.01	8	0
Chick embryonic brain RNA:			
Unbound RNA	11.00	8	0
Bound RNA	0.25	8	0
Bound RNA	0.50	8	0
Rat liver RNA:			
Unbound RNA	10.00	8	0
Bound RNA	0.03	8	0
Bound RNA	0.60	8	0

TABLE I cont.

	A_{260}[a]	*N° of PNP Used*	*N° of PNP Used*
Calf kidney RNA:			
Unbound RNA	12.000	8	0
Bound RNA	0.25	8	0
Bound RNA	0.50	8	0
δ-crystallin mRNA	0.34	6	0
Rous sarcoma viral RNA	0.90	6	0
Rabbit macrophage RNA	3.00	6	0
Bombyx mori rRNA	15.00	6	0
Bombyx mori tRNA	12.00	16[c]	0
E. coli rRNA	12.00	9	0
E. coli tRNA	12.00	21[c]	0
Total chich embryonic RNA (DNase treated)	15.00	3	2
Total chick embryonic RNA (pronase treated)	15.00	3	2
poly (A)	0.005	9	0
poly (A)	0.01	9	0
poly (A)	0.05	6	0
poly (A)	0.10	6	0
poly (C)	0.09	3	0
poly (G)	0.09	3	0
poly (I)	0.10	3	0
Control (without RNA)	—	57[c]	0
Control (with carrier RNA)	15.00	90[c]	0

Culture technique, addition of RNA, incubation conditions, enzymic digestions and heat treatment are all described in detail elsewhere (20).

[a]The final concentration was adjusted to 15 A_{260} per ml by addition of carrier RNA. *E. coli* or *Bombyx mori* rRNA and tRNA, in a ratio of 10:1 was used as carrier RNA.

[b]Initial scoring of the differentiated explants was based on the beating property of the PNP cells. The beating and the non-beating explants were pooled separately and then divided randomly into batches which were then subjected to ultra-structural histochemical and biochemical examinations as described earlier (20).

[c]The number of explants represents a cumulative total of several experiments.

at 0.0005 A_{260} units/ml, which represents about 60-fold purification over the bound II CEH-RNA. We attempted to purify the fraction 1 'a' RNA further by a second electrophoretic run after heating the RNA in 70% formamide at 80°C for 3 min followed by a rapid cooling before applying to 7.5% polyacrylamide gel in 98% formamide and by prolonging the electrophoresis to 12-14 hrs. This resulted in separation of the fraction 1 'a' RNA into a slow-moving minor spot and a faster moving major spot (Fig. 5). When the two spots were eluted from gel, passed through oligo (dT) cellulose column as before and tested for their activities to induce differentiation in PNP, the fast-moving spot exhibited a better inducing activity (68%) compared to the slower moving spot (24%) at identical concentrations (0.005A_{260}/

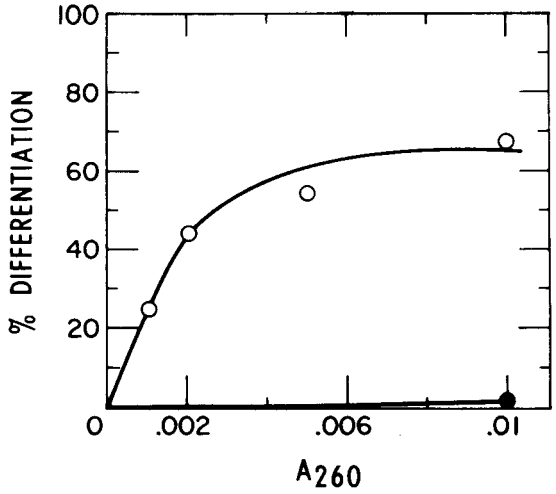
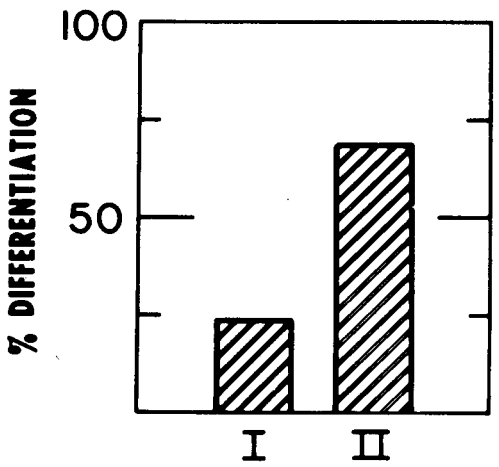
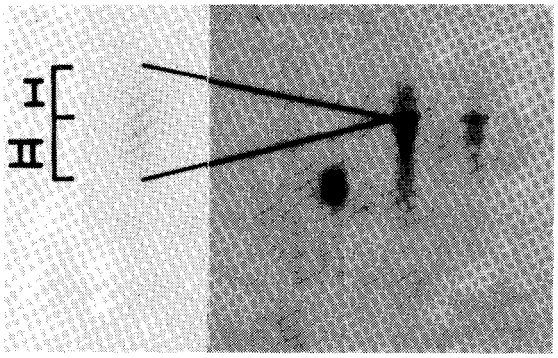


Fig. 4. Effect of varying amount of CEH-RNA fraction 1'a' on PNP. The PNP explants were cultivated in the presence of varying amount of the inducer RNA and examined for differentiation after 96 hr of incubation as described in text and elsewhere (22). A minimum of 18 and a maximum of 38 explants were used for each point. ○, untreated RNA; ●, RNA, pretreated with insoluble pan RNase (see ref. 22).





ml). The fast-moving spot migrated as 7S like material on 7.5% polycrylamide gel in formamide (Fig. 6) and had identical base composition as fraction 1 'a' RNA and will therefore be referred from here on as 7S CEH-RNA.

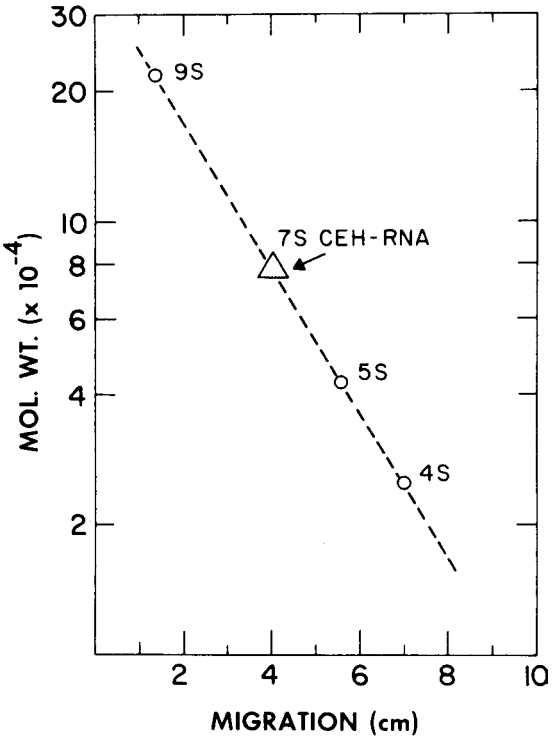


Fig. 6. Relationship between molecular weight and electrophoretic mobility. Electrophoresis of 7S CHE-RNA was done, as in Fig. 5, on 7.5% polyacrylamide gel in for ma mide, in presence of marker 4S, 5S and 9S RNAs.

The nucleoside composition of the fraction 1 'a' RNA was determined after post-labeling the RNA with potassium borotritiide according to Randerath (25,26). The RNA was unusually rich in adenylate residues. The presence of 54% A would suggest that about one-

Fig. 5. Further purification of CEH-RNA: The bound II fraction 1 RNA from the sucrose gradient centrifugation as above was run first on 7% polyacrylamide gel in formamide as in Fig. 2. The top band (fraction 1'a-) was eluted from the gel, passed through oligo(dT) cellulose, and run again on 7.5% polyacrylamide gel in 98% formamide for 12 hr at room temperature. The RNA was heated to 80°C for 3 min in 79% formamide and rapidly cooled in ice-cold H_2O before placing on gel. The RNA was then eluted from gel areas I and II, as indicated, passed through oligo (dT) cellulose again, and tested for its inducing effect as before (20, 22).

third of the RNA is comprised of poly(A) stretch or stretches (Table II). Alternatively, the RNA may contain some contaminant poly(A) fragment(s) of sizes that could not be resolved under the separation technique used. The A rich nucleoside composition would permit little double strandedness in the molecule. The 5S like bound RNA (fraction 'b + c') was even more rich in poly(A). There were, however, no known modified nucleosides present in either RNAs. An unidentified spot N' moving slightly above uridine trialcohol was occassionally observed in 7S RNA (Fig. 7). The other unidentified spot N' moving slightly above guanosine

TABLE II

Nucleoside composition analysis

Nucleoside	*CEH-RNA Bound II Fractions*		*$tRNA_1^{Val}$*
	1 'a'	*1 'b + c'*	*(E. coli)*
	(7S)	*(5S)*	
U'	16.0	10.7	10.6
A'	53.6	70.8	14.4
C'	15.2	9.2	24.3
G'	15.1	9.2	22.8

Nucleoside composition analysis was done according to Randerath (25) and as described earlier (22).

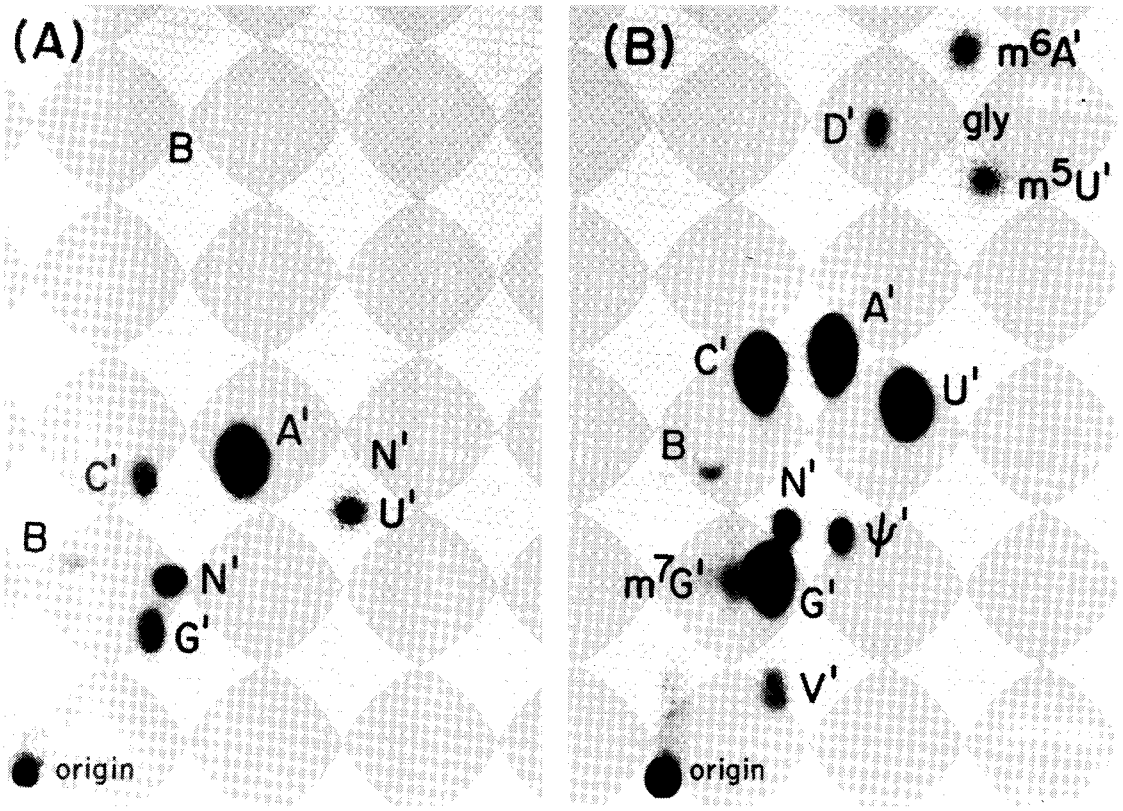


Fig. 7. Autoradiograph map of [^{3}H] labeled digests of CEH-RNA fraction 1 'a' RNA and *E. coli* $tRNA_1^{Val}$. For details of borotritiide reduction after digestion of the RNAs and separation techniques and other details see ref. 22. (a) CEH-RNA fraction 1 'a'; (b) *E. coli* $tRNA_1^{Val}$.

trialcohol observed in both 7S RNA and $tRNA_1^{Val}$ is believed to be inosine produced during the reduction procedure (W. Schmidt, personal communication). Preliminary experiments on terminal analysis based on reduction with borotritiide indicated that the 3'-terminus is adenosine and the same experiment also suggested that the 5'-terminus may not be capped. Experiments to verify these results are currently in progress.

Characterization of Differentiation

The PNP cultivated in presence and in absence of RNA (or with carrier RNA) added to the medium were examined at regular intervals for changes in growth and morphology and appearance of pulsating tissues. None of the PNP cultivated in the absence of competent RNA contained beating tissues and showed the specific changes described below. The AP, which is known to contain the presumptive heart forming region (see ref. 20) on the other hand, developed into a complete embryo with rhythmically pulsating heart, as was observed by earlier workers (27, 28). The growth pattern of the control PNP was similar to that described earlier (19). The primitive streak disappeared after 24 hr and no axial structures or twitching tissues were observed even after 10 days of incubation. In contrast, the addition of competent CEH-RNA fractions resulted in the formation of distinct heart-like tubular structures capable os spontaneous and rhythmic pulsations after 4 days of incubation.

Since the morphology of cells in culture is known to be influenced by culture medium, growth conditions, intercellular contact, etc. (29, 30), and the ability of cells to beat is influenced by extracellular ionic concentration and culture conditions (31), we attempted to redefine the PNP differentiation by the following criteria in addition to changes in morphology and acquisition to rhythmic pulsations: i) the presence of striated myofibrils; ii) the presence of glycogen particles; iii) the appearance of actin and myosin-like polypeptides, iv) an increased level of acetylcholinesterase activity. For examining these changes, we have used a large number of randomly selected beating and non-beating (without RNA or with carrier RNA alone) PNP. Double-blind control experiments were also conducted, on occasion, for scoring the beating PNPs. It must be pointed out that none of about 300 PNPs of control series examined ever exhibited pulsations even after prolonged incubation.

i) *Appearance of myofibrils and glycogen granutes:* It is well established that appearance of myofibrils and glycogen particles are two identifying characteristics for differentiation of early embryonic chick heart muscle cells *in vivo*, in cell suspensions, and *in vitro* (31, 32). Ultrastructural examination of RNA-treated and control PNPs showed scantily dispersed endoplasmic reticulum, nuclei, mitochondria, Golgi complex and other normal cell components, in both the PNPs, but the beating PNPs contained highly differentiated myocytes similar in ultrastructural complements to normal embryonic myocardial cells (32). Clearly evident in Fig. 8 were the orderly arranged myofibrils with thick and thin filaments and Z-bands which were notably absent in all the control PNPs examined.

Because of its localization in cardiac muscle cells of young chick heart in abundance glycogen has been used as a marker for young heart muscle cell differentiation (31, 33). Histochemical examination of chick embryonic heart and the PNPs by PAS staining method (Fig. 9) revealed that the hearts and the beating PNPs were rich in glycogen particles, whereas the control non-beating PNP lacked the PAS positive material. The relatively slight localization of PAS positive material in the peripheral region or the control PNP is typical of embryonic endodermal cells (34). Treatment of the PNP with malt diastase abolished the PAS staining reaction suggesting that the PAS-positive material was glycogen.

ii) *Synthesis of actin* and *myosin-like proteins in PNP:* The major constituents of myofibrils are the proteins, myosin and actin, organized into interdigiting filaments. When the PNPs were labeled with [^{3}H]-alanine, there was a 2.5 fold increase in myosin obtained from differentiated PNP compared to the proteins of control PNP under identical conditions. The incorporation of label into total TCA-insoluble proteins was 1.7 fold higher in the beating PNP than in control non-beating PNP. Figure 10 shows an examination of the partia-,

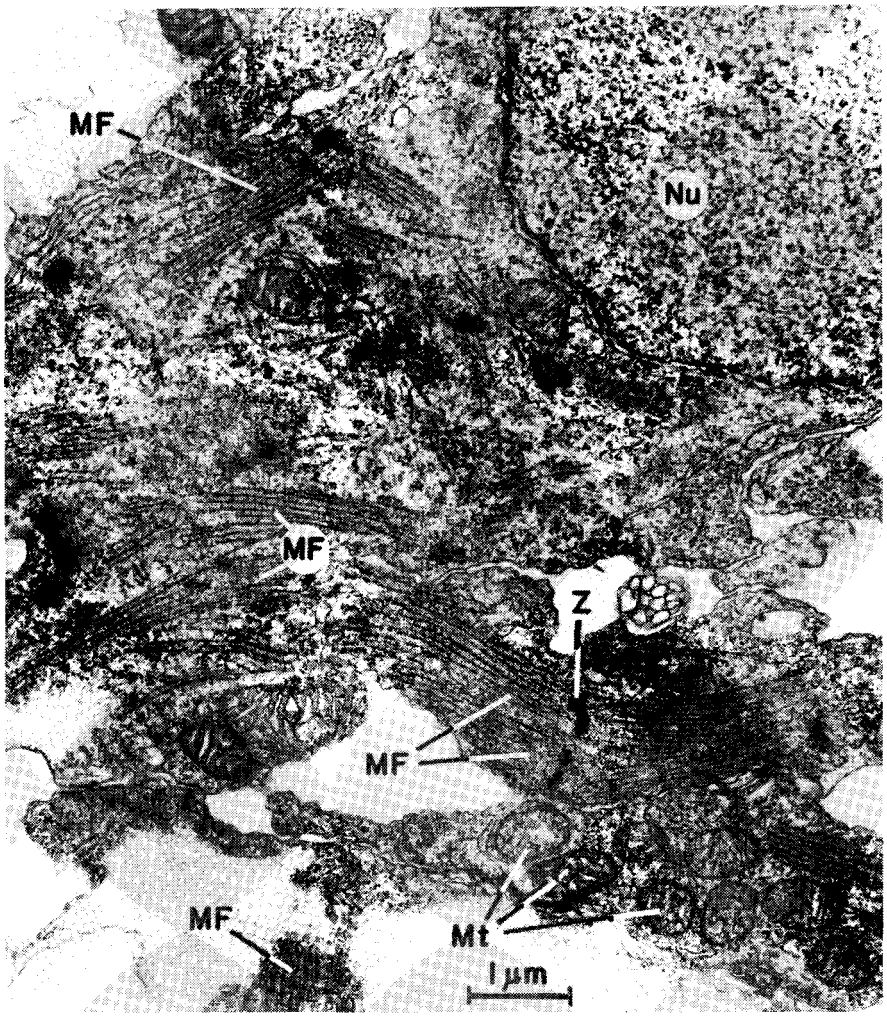


Fig. 8. Electron micrograph of RNA-treated PNP cells. The PNP explants were cultivated in presence of inducer RNA and examined for ultrastuctural components as before (20). MF, myofibrils; Nu, nucleus; Mt, mitochondria; 2, 2-band.

lly purified labeled muscle proteins on SDS-acrylamide gels. Prominent peaks similar in electrophoretic mobility to authentic actin and myosin markers were seen in all the PNPs that contained beating tissues. The proteins extracted from the control PNP also appeared to contain actin and light-chain myosin-like polypeptides, but the level of [^{3}H] alanine incorporation was lower. Similar results were obtained when [^{3}H] lysine and [^{35}S] methionine were used to label the proteins, although relative levels of the label in peaks differed based on the amino acid used. It must be pointed out here that acting and myosin-like polypeptides are also found in several non-muscle and premyogenic cells even though no myofibrillar structures are present (35, 36).

iii) *Acetylcholinesterase activity:* The myogenic process involves, in addition to increased synthesis of muscle specific proteins, elaboration of specialized membrane components including the enzyme acetylcholinesterase (AChE), which mediates transmission of impulse from nerve to muscle (37, 38). Cells of myogenic origin differentiate in culture in absence of neuronal elements produce AChE and acetylcholine receptors similar to muscle tissue *in situ* (39). In differentiating chick

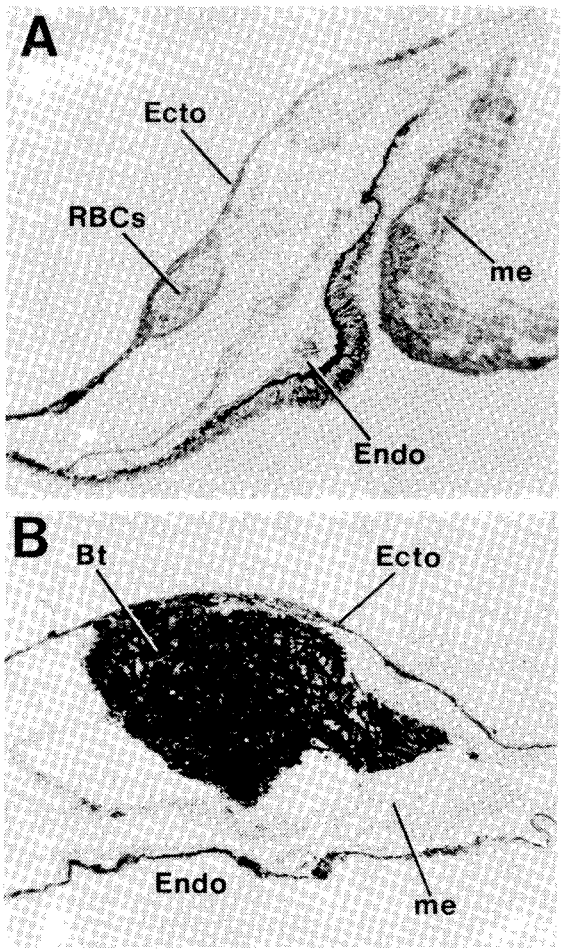


Fig. 9. Photomicrograph of PNP cultivated in presence and in absence of inducer RNA after PAS staining for glycogen. For details of incubation, culture techniques, and staining reaction see ref. 20. A. beating PNP treated with the inducer RNA; B. non-beating PNP (control). Ecto, ectoderms; RBC, red blood cell; me, mesenchyme; Endo, endoderm; Bt, beating area (which is also PAS positive).

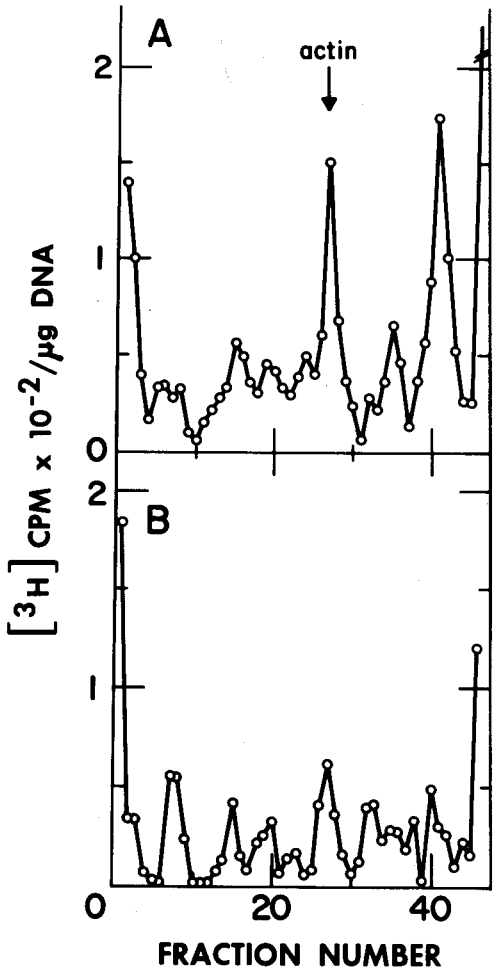


Fig. 10. SDS-polyacrylamide gel electrophoresis of PNP muscle proteins. The PNPs were labeled with [^{3}H] alanine added directly to the medium with or without inducer RNA. The proteins were extracted and examined on 5.6% polyacylamide gel as before (22). Purified myosin and actin from the chick embryonic heart were run simultaneously as markers. The top stained gel indicates purified myosin bands and arrow indicates the position of purified actin. Fraction 1 represents the bottom of gel.

muscle cells in culture, for example, AChE activity appears concurrently with acetylcholine receptors and the synthesis is coordinated with other muscle membrane components (38). It has been possible to localize AChE histochemical techniques utilizing the "direct coloring" thiocholine reaction of Karnovsky and Roots (40) and to quantitate the enzymatic activity by biochemical assay monitoring the hydrolysis of labeled acetycholine according to Nirenberg and coworkers (41). When the PNPs were cultivated in presence of the induce CEH-RNA to allow differentiation as indicated above and the homogenate examined for its activity, there was a 3.4 fold increase in the rate of hydrolysis in the homogenate of beating PNP compared to that of non-beating PNPs (42) and a 6-fold increease over that of unincubated PNP. The increase in AChE activity was always found in the same batches of PNP that also exhibited the specific morphological and biochemical changes, whereas the control PNP grown under identical conditions with the exception of the inducer CEH-RNA distinctly lacked these properties.

Histochemical examination for acetylcholinesterase activity in the early stages of chick development was recently undertaken in our laboratory using Karnovsky and Root's method of staining (40). The advantage of this method is that the color is produced at the site of enzymatic activity thus providing a clear visualization of the locale of the enzyme during various phases of development. The PNP explant was stained *in toto* after incubation for 96 hr in presence of CEH-RNA and the whole mounts were embedded in paraplast and sectioned serially. When observed under oil immersion optics AChE positive cells revealed the presence of yellowish brown granules; the same sections under phase-contrast optics appeared dark brown. Figure 11 shows the distribution and localization of these granules in both differentiated and undifferentiated cells of PNP. There were clear differences in localization of grain distribution in the differentiated (beating) and undifferential (control) PNP and the cells of beating PNP contained significantly larger deposition of grains compared to the control cells. Similar examination of the unincubated PNP of stage 4 blastoderm showed that the stage 4 cells contain grains similar in intensity and distribution to the incubated control PNP, but the grains were mainly localized in the epiblast.

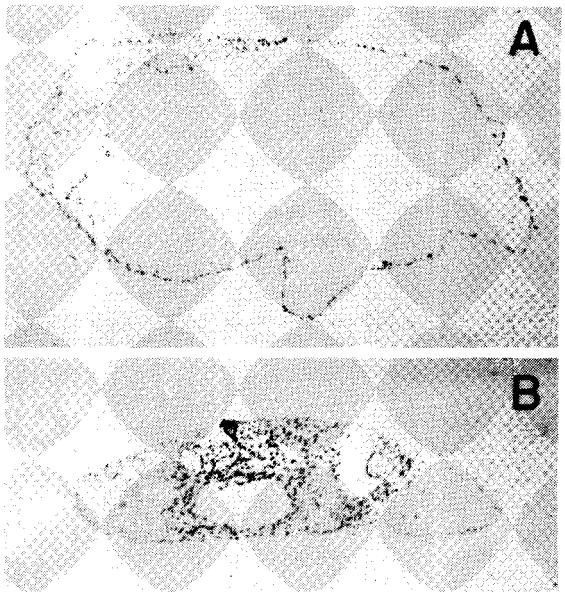


Fig. 11. Transverse sections of PNP after histochemical examination for acetylcholinesterase. The PNP explants cultivated in presence (B) and in absence (A) of inducer RNA were stained *in toto* for acetylcholinesterase activity (21), sectioned at 5 μM thickness and examined under light microscope. The grains indicate the presence of acetylcholinesterase.

The Dependence of Differentiation on Inducer RNA

As indicated in Table I none of the PNPs cultivated in absence of the inducer RNA (or in presence of carrier RNA alone) exhibited rhythmic pulsations or any of the other specific changes characteristic of myogenic differentiation. In contrast, the addition of total RNA isolated from the 16-day old chick embryonic heart (CEH-RNA) or the active fractions obtained during purification of CEH-RNA caused a specific mode of morphological and biochemical changes in the explants. Initial scoring of differentiated explants was based on the appearance of rhythmic beatings of the cells. The beating explants also contained highly ordered myofibrillar structures and synthesized increased levels of the contractile proteins, actin and myosin. The appearance of abundant glycogen deposits and high level of acetylcholinesterase activity were additional markers indicating the transition to heart-like differentiation. Treatment of RNA with pancreatic and T_1 ribonuclease abolished the inducing capacity totally, whereas DNAse and pronase treatment had no effect. Following pancreatic RNase digestion, the mixture was either phenol extracted, ethanol precipitated in presence of carrier RNA and then examined for its effect on PNP or when insoluble pancreatic RNase was used the mixture was first freed of the enzyme by centrifugation and the supernatant was used. In both cases, the activity was totally abolished. The RNA inducing action was sensitive to alkali but resistant to heat treatment. The oligo(dT) cellulose bound RNAs obtained from the chick brain, calf kidney and rat liver under identical conditions did not induce the differentiation when examined as above, neither did the RNAs from the silkgland of *Bombyx mori, E. coli,* δ-crystallin

mRNA, rabbit macrophage RNA, and Rous sarcoma viral RNA. The addition of the latter three RNAs did not lead to synthesis of the RNA specific proteins in measurable amounts. Synthetic polynucleotides, added at various concentrations, were also not effective.

Thus, it would appear that the induction of the pattern of changes observed in the PNP cells was indeed dependent upon the presence of an RNA species in the culture medium. Recently, we have found that the low molecular weight fraction of the total RNA obtained from the leg muscle of the 16-day old chick was also capable of causing spontaneous and rhythmic pulsations in the PNP, although it is not known yet whether other parameters of cardiac differentiation were also present.

Uptake of Inducer RNA

In previous studies on RNA-mediated biological transitions in mammalian cells (11), it seems there is no clear evidence for the uptake of RNA in its native form. Neither is the mechanism for the uptake known. In an attempt to investigate whether the 7S CEH-RNA added to the culture medium was indeed taken by the PNP cells, we labeled the RNA with [^{125}I]-iodine to a high specific activity (4.2×10^7 cpm/ μg, unpublished observations) which was then added to chick Ringer's solution containing previously disaggregated PNP cells. After an incubation for 60 min, the cells were spun down and washed repeatedly until no more label was recovered in the washes. The cells were then homogenized and the RNA was extracted with phenol : isoamyl-alcohol : chloroform, as usual, in presence of carrier RNA. The RNA thus recovered was then examined on 7.5% polyacrylamide gel in formamide (Fig. 12). It is clear that the RNA recovered after several washes of the cells is identical, in its mobility on the gel, to the RNA added to the medium. After eluting from the gel, the RNA was also subjected to a T_1-RNase fingerprinting analysis. Preliminary results (not shown here) indicated that the fingerprint patterns of the two RNA sports were similar. The extent of RNA uptake after one hour incubation varied from experiment to experiment. Based on

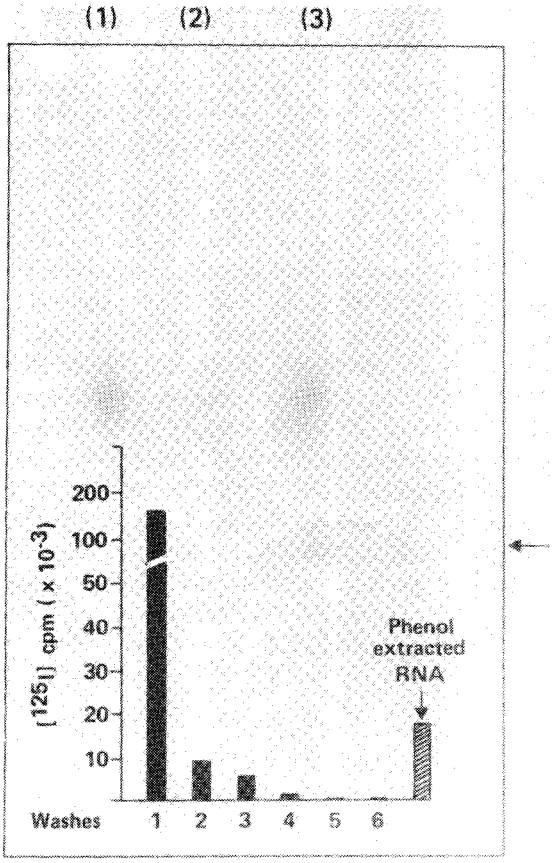


Fig. 12. Electrophoresis of [^{125}I] CEH-RNA recovered from PNP cells. 7S CEH-RNA was labeled with [^{125}I] according to the method described by Commerford (62) and to be reported elsewhere. The labeled RNA (470,000 cpm) was added to 0.5 ml of chick Ringer's solution (20) containing about 270,000 cells of PNP obtained after mild disaggregation of stage 4 PNP. The cells were incubated at 37°C for 60 min and then removed by centrifugation for 8 min at 2,000 rpm. The cells were washed repeatedly with Ringer's solution until no more counts were recovered (see bottom part of figure). RNA was then extracted from the PNP cells in presence of 2.0 A_{260} units of *E. coli* tRNA added as carrier as before (22). An aliquot of the RNA recovered from the PNP cells was run on 7.5% polyacrylamide gel in formamide along with the labeled 7S CEH-RNA as in Fig. 5. Labeled RNA spots on gel were detected by exposure to X-ray film Kodak XR-1. (1) and (3), [^{125}I] 7S CEH-RNA; (2) [^{125}I] RNA recovered from PNP cells.

CCl_3COOH-insoluble cpm 0.9 to 3.8% of the input was recovered from the PNP cells. The reason for this variation is not presently known. In order to localize the RNA in PNP, the cells were sectioned to 5 μm thickness and autoradiographed. Although the results of this

experiment is not yet available, a similar experiment utilizing [^{32}P] labeled RNA indicated that silver grains were distributed inside the cell with no apparent preference to nuclear or cytoplasmic regions. The number of grains per unit area was six-fold higher when labeled oligo(dT) cellulose bound RNA was used compared to the unbound RNA.

Mode of Action of 7S CEH-RNA

Since the 7S CEH-RNA was purified from the total oligo (dT) cellulose bound RNA fraction, it was reasonable to assume that this RNA might be translatable, although the product of translation must be a small polypeptide. Addition of RNA, at various concentrations, to the rabbit reticulocyte cell-free extracts treated with nuclease (42) did not result is any stimulation of CCl_3COOH-insoluble material. On the contrary, the RNA effectively inhibited globin mRNA and rat liver poly [A(+)] RNA-dependent translations *in vitro*. This suggested that the observed biological activity of 7S CEH-RNA in the PNP might be related, in some way, to an effect on translation process *in vivo*. If this is so, then it must also affect the *in vitro* utilization of mRNA from the same source from which the inhibitor 7S RNA was originally isolated. Fig. 13 and Fig. 14 illustrate that globin mRNA as well as the total chick heart poly [A(+)] RNA-dependent translations in rabbit reticulocyte lysates were inhibited effectively at various concentrations of the inhibitor 7S CEH-RNA. Addition of 5S rRNA of *B. mori* at comparable concentrations, on the other hand, had no effect. The traslational activity of globin mRNA was reduced by about 87% when 1.8 μg of 7S CEH-RNA were present in the incubation mixture. This represents a 40-fold molar excess of 7S CEH-RNA over globin mRNA, assuming an approximate molecular weight of 80,000 for 7S CEH-RNA based on its electrophoretic mobility on 7.2% polyacrylamide in 98% formamide. However, the inhibition by 7S CEH-RNA of globin mRNA translation could not be overcome by increasing the concentration of globin mRNA in the reaction mixture. The

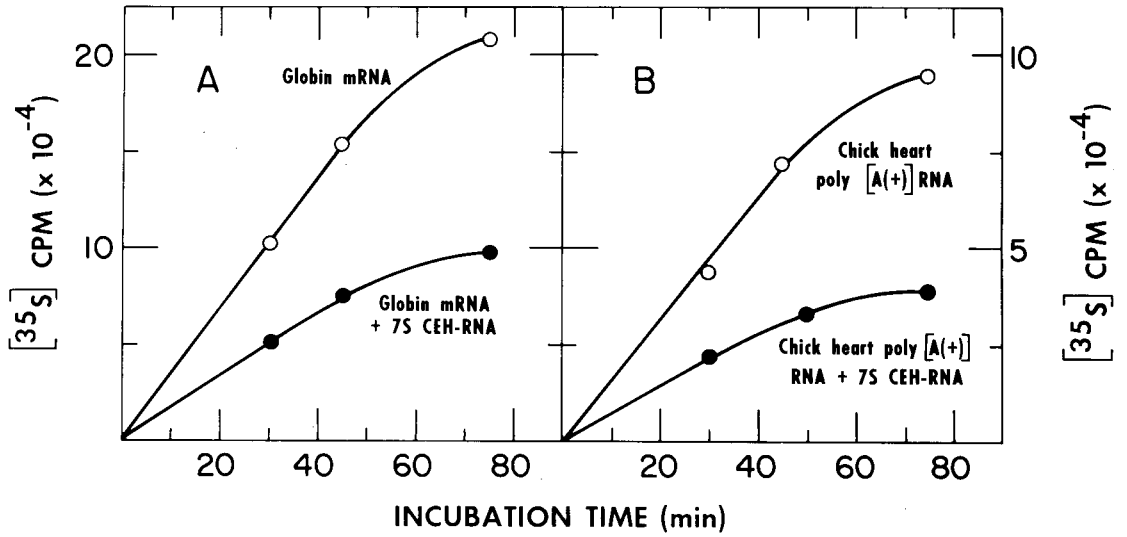


Fig. 13. Effect of 7S CEH-RNA on translation of globin mRNA and chick embryonic heart poly [A(+)] RNA. Translation of globin mRNA (A), 2.2 μg/ml was done in rabbit reticulocyte lysate after nuclease treatment (63,64) in presence of 0.9 μg of 7S CEH-RNA added to each assay tube at the start of incubation. Aliquots, 5 μl each, were removed at time indicated. Note that the concentration of 7S CEH-RNA used was effective for about 50% inhibition of translation only (see also Fig. 14). ○, control; ●, mRNA plus 7S CEH-RNA. (For details see ref. 64).

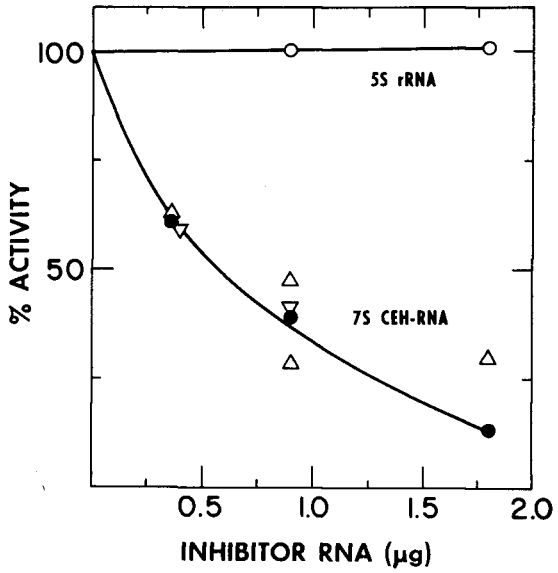


Fig. 14. Inhibition of translation by 7S CEH-RNA at varying concentrations. Translation of globin mRNA, 2.2. μg/ml, and chick heart poly[A(+)]RNA, 20 μg/ml, was done, as in Fig. 13 in presence of varying concentrations of 7S CEH-RNA or 5S rRNA. Aliquots were taken after 30 min of incubation at 30°C. The effect of 5S rRNA of the posterior silkgland of *B. mori* (65) was tested for globin mRNA translation alone. ●, 7S CEH-RNA plus globin mRNA; 0, 5S rRNA of *B. mori* plus globin mRNA; △ or ▽, 7S CEH-RNA plus chick heart poly [A(+)]RNA (△ and ▽ represent two separate experiments).

lack of stoichiometric relationship between the mRNA and the inhibitor RNA would suggest that the latter is not similar, in its mechanism of inhibition, to the myosin mRNP-tcRNA of the chick embryonic leg muscle described by Heywood and coworkers (44, 45). The 7S CEH-RNA apparently competes with some component in the rabbit reticulocyte lysate, since increasing the amount of lysate in reaction mixture diminished the inhibitory effect of 7S CEH-RNA (unpublished results).

The previously described low molecular weight RNAs implicated in the control of translation are rich in one or other nucleoside content. Heywood's tcRNA from chick leg muscle, for example, contains 48% U (44), Bogdanovsky's rabbit reticulocyte RNA contains 46% A (46, 47), the inhibitor RNA from *A. salina* has 47% U and the activator RNA from the same source contains 51% G (47). Since the 7S CEH-RNA described in these studies is rich in A (53%) and contains poly(A) tracts, we deemed it necessary to ascertain whether the inhibitory effect of the RNA could possibly be due to the poly (A) tracts alone. Single stranded poly(A), poly(U), poly (I) and double stranded RNA have previously been shown to cause inhibition of cell-free translations (48-50). For this purpose, the 7S CEH-RNA was first digested with insoluble pancreatic RNase and the supernatant obtained after centrifugation which should contain the undigested poly(A) fragment(s), was tested for globin mRNA translation. As indicated in Fig. 15A, the nuclease digested material was as active in inhibiting translation as the control. Under the same conditions of digestions [^{32}P] 5S RNA of *B. mori* became 95% CCl_3COOH soluble. Digestion with micrococcal nuclease, on the other hand, resulted in a significant loss of inhibition due to RNA (Fig. 15B). The inhibitory effect, in fact, was mimicked by synthetic $poly(A)_{11\text{-}19}$, which when added to the translation mixture at comparable concentration caused an inhibition of amino acid incorporation, and treatment of poly(A) with micrococcal nuclease totally diminished the inhibition. These results, therefore, suggested that the 7S CEH-RNA inhibition of translation, for at least globin mRNA, might be due to the poly(A) tract(s) present in the RNA. Poly (A) also inhibited the traslation of total chick embryonic heart poly[A(+)]RNA as well as that of globin mRNA (see Fig. 16). It is possible however, that the inhibition of chick heart poly[A(+)]RNA due to 7S CEH-RNA, at least at a specific concentration, may be selective for some fraction of translatable mRNA in the total chick embryonic heart poly[A(+)]-RNA population and the effect produced by synthetic poly(A) (or by the digestion product of 7S CEH-RNA) may not be the same. We, therefore, examined the translational products of the chick heart poly[A(+)]RNA at several concentrations of 7S CEH-RNA and synthetic poly(A) on 10% polyacrylamide gels in SDS (Fig. 16). The pattern of labeled polypeptides obtained after 30%, 50% and 70% inhibition of translations due to the two RNA were identical. All bands appeared to be inhibited without an apparent difference in intensity of label or their migration properties. The pre-

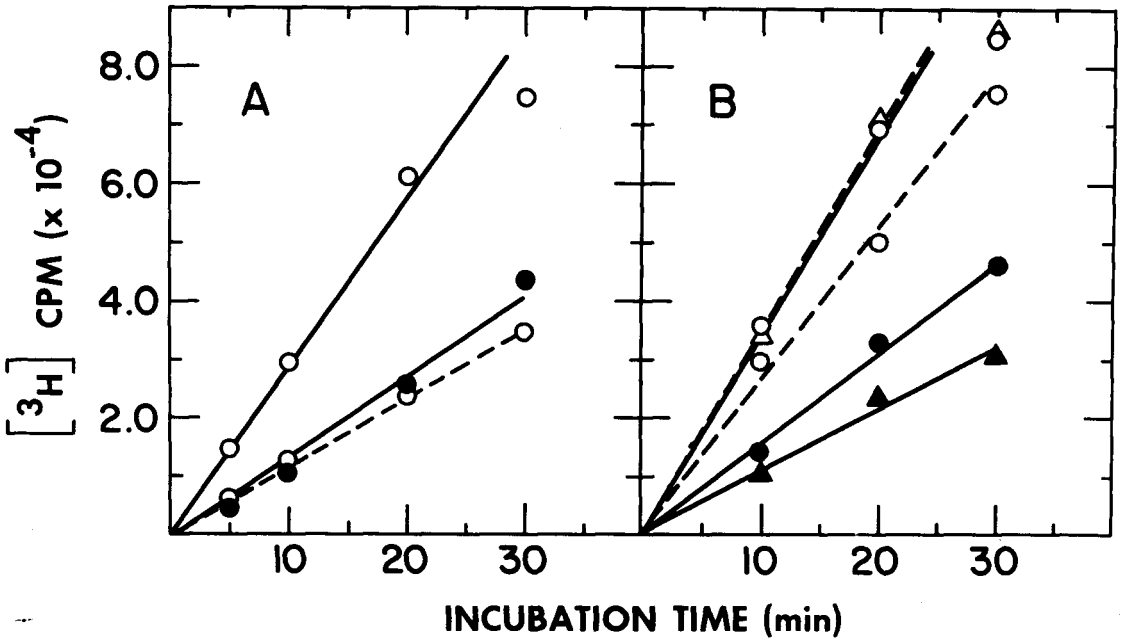
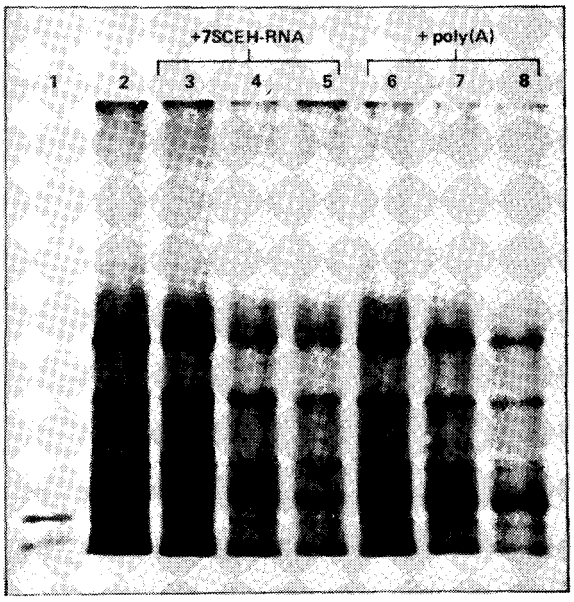


Fig. 15. Effect of RNase treatment of inhibitor RNA on globin mRNA translations. Translation of globin mRNA was done in standard incubation condition using 10 μCi [^{3}H]leucine as in Fig. 13. 7S CEH-RNA, 0.64 μg, or synthetic poly(A), 0.4 μg, alone or after digestion with nuclease as described previously (64) were added at the start of incubation and radioactivity in 5 μl aliquots was measured at times indicated. (A) effect of pancreatic RNase digestion of 7S CEH-RNA on globin mRNA translation; 0, control; • plus 7S CEH-RNA; 0——0, plus 7S CEH-RNA after pan RNase digestion. (b), effect of micrococcal nuclease digestion of 7S CEH-RNA or of synthetic poly(A) on globin mRNA; 0, control; • plus 7S CEH-RNA; 0——0, plus 7S CEH-RNA after micrococcal nuclease digestion; Δ plus poly (A), Δ——Δ, plus poly (A) after nuclease treatment. (For details see ref. 64).



sumptive heavy chain myosin, that should migrate with a mobility of about 200,000 mol. wt., was made in considerably less amounts than expected in both inhibited and uninhibited mixtures. In most translation assays the

Fig. 16. Polyacrylamide gel separation pattern of labeled translational products of chick heart poly[A(+)] RNA in presence or absence of inhibitor RNA. Translation of chick heart poly[A(+)] RNA, 20 μg/ml, was done in standard incubation conditions with or without 7S CEH-RNA or poly(A) and the labeled were processed and applied to gel as described earlier (64). Electrophoresis was done according to Laemmli (65) and labeled bands were located by fluoragraphy (67). Non-labeled marker proteins were run simultaneously on the same gel and located by staining with Coomasie blue. (1), control nuclease treated lysate; (2), chick heart poly[A(+)] RNA; (3), (4), and (5) contain poly [A(+)] RNA plus 0.32, 0.64, 1.1 μg of 7S CEH-RNA respectively; (6), (7) and (8) are the same as (2) but contain 0.23, 0.46, 0.92 μg of poly(A) respectively. (For details see ref. 64).

heavy chain myosin-like band was totally absent, although bands in the region of 100,000 to 150,000 mol. wt. were visible. We do not, at present, know whether this is due to the possible degradation of myosin heavy chain or its incomplete synthesis. It is, therefore, difficult to evaluate the specificity of inhibition, if any, by 7S CEH-RNA for the myosin heavy chain mRNA translation.

The relationship between the 7S CEH-RNA induced biological transition in the PNP and the inhibition of *in vitro* translations due to the RNA is not totally clear. Since the product of pancreatic RNase digestion of 7S CEH-RNA results in a loss of its inducing activity and the fact that synthetic poly(A) alone, at several concentrations, cannot reproduce the effect of 7S CEH-RNA on PNP, it would be reasonable to assume that a control at the level of translation, based on the observations on *in vitro* translational assays, can be eliminated as one of the possible mechanisms of action of the RNA on PNP. However, a mechanism envisaging a selective discrimination of specific mRNA translation under distinct physiological conditions, which are not necessarily duplicated by rabbit reticulocyte lysates, cannot be excluded. A control at translation level is reported to exist in the primary myoblast cell culture, the terminal differentiation of which, with respect to musle specific protein synthesis, is preceded by stabilization of myosin mRNA and its subsequent translation (51) Such a stabilization of mRNA can be visualized by way prevention of specific mRNA translations *in vivo* due to an inhibitor RNA molecule. It was suggested that the inhibition of protein synthesis due to RNA in *A. salina* is overcome by an activator RNA molecule, thus permitting the onset of protein synthesis in developing embryo. Whether such a molecule exists in chick embryonic heart tissue remains to be seen.

Conclusiones

During the past 50 years or so embryologists have focused their attention on induction and activation which is interpreted as processes whereby the course of embryonic development is altered as a result of information received or lost. It is now generally believed that factor(s), diffusible and possibly macromolecular in nature, are involved in some way in bringing about the changes in embryogenesis. The nature of the factor(s) and their mode of action remains unknown. The involvement of RNA, and/or protein translated from it, as regulatory elements during embryonic development has been postulated as part of a model on control of eukaryotic gene expression by Britten and Davison (3-5). Although there is no clear and direct experimental evidence to our knowledge in support of the main theme of the model, the control of gene expression by cytoplasmic and extracellular factors in general is a well known phenomenon. The requirement of non-histone proteins in control of gene expression in reconstituted chromatin (Stein and Stein, this issue), the migration of proteins and RNA between cytoplasm and nucleus and their specific association with chromosomes at distinct stages of development (12, 52, 53), and the case of a protein in Mexican axolotl synthesized during oogenesis which seems to be essential for activation of nuclear genes required during gastrulation and organogenesis (17), have been documented.

Substantial evidence also exists on the transfer of RNA between different kinds of cells during specific immune responses (54) and on the ability of foreing RNA to induce specific immune response (55). Small molecular weight nuclear RNA (SnRNA) ranging from 65-200 nucleotides in chain length have been found in nuclei, and occassionally in cytoplasm, of several eukaryotes (56, 57). These RNAs have been characterized structurally and a few have even been sequenced. Since these RNAs are probably too small to serve as mRNA and their relation with the large heterogenous RNA (HnRNA) is not defined, it appears that the SnRNAs comprise a separate class of RNA with no known function, although in some cases they are implicated in specification of chromosomal functions (57). Another form of low molecular weight RNA, chromosomal RNA (cRNA), has been described in ascites cells (58). The cRNA is reported to represent a fraction of the repetitive sequence present in the HnRNA. The functional significance of this RNA remains unknown. Erikson reported (59) that a 7S RNA, identical in structure to that associated with the avian oncornaviruses, is present in low

concentrations in normal uninfected chick cells. Structural analysis of the RNA shows a relatively high A and G content but the fingerprint profile of the RNA reveals that it does not contain poly(A) stretches. RNAs similar in structural features to the 7S RNA of chick cells are also found in several viral preparations and in normal mammalian cells (60, 61), the function of which is not clear.

Two classes of low molecular weight RNA (tcRNA) involved in control of translation were reported to be present in embryonic chick leg muscle (45, 46). One such RNA is known to contain approximately 50% uridylate residues and have a mol. wt. of about 10,000. The RNA also inhibits effectively the *in vitro* translational activity of poly(A) containig mRNA. Their physiological role in translation process, however, remains enigmatic. Ochoa and coworkers (47) recentiy reported the presence of two low molecular weight RNA species in the embryos of *Artemia salina*. One of these RNA is a translational inhibitor of about 6000 dalton in size and is rich in pyrimidines (47% U); the other, an activator RNA is able to complex with the inhibitor to neutralize its inhibitory activity. The RNA, therefore, can play a regulatory role causing the onset of protein synthesis in the developing embryos.

In a recent communication (64), we reported that the 7S CEH-RNA is capable of inhibiting the *in vitro* translation of both homologous and heterologous mRNAs effectively. The inhibition is non-competitive with respect to mRNA and is overcome by some factor(s) present in the rabbit reticulocyte lysate. The inhibition, however, appears to be effected by the adenine-rich segment(s) of 7S CEH-RNA. It would be of interest, therefore, to examine whether the translation inhibitory effects of previously reported tcRNA can be discriminated from those likely to be produced by the corresponding nucleotide-rich polynucleotides. We do not at present know whether 7S CEH-RNA has a specific effect on transcriptional activity of the PNP cell. Experiments designed to investigate this possibility with isolated nuclei are currently in progress.

Thus, we can summarize briefly with the statement that inspite of several examples for both RNA and proteins as candidates for regulatory elements in gene expression, there is no clear definition of their role in normal cellular processes. The finding that the 7S CEH-RNA can produce a specific mode of change characteristic of embryonic cardiogenic process offers first such opportunity to test the role of an RNA in communication of regulatory information in an experimentally analyzable biological system. Our present experiments are also designed to investigate whether the 7S RNA can be detected in measurable amounts at relevant stage(s) and areas in the chick embryo during development so that the physiological role of the RNA in heart induction can be understood.

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