Effect of cytosol on transport of RNA *in vitro* during cardiac hypertrophy

Shanmugam MEENAKSHI, Chellaiah THIRUNAVUKKARASU and Chellam RAJAMANICKAM*
Department of Biochemistry, School of Biological Sciences, Madurai Kamaraj University, Madurai 625 021,
India

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An enhanced RNA-transport activity was observed *in vitro* from nuclei obtained from animals with cardiac hypertrophy as compared with that of sham-operated controls. The 100000g supernatants obtained from hypertrophic hearts stimulated the RNA transport from nuclei of sham-operated controls, and this stimulation was maximum with 40% supernatant. Ca^{2+} - and nucleic acid-dependent ATPase and alkaline phosphatase activities, which may be involved in an energy-dependent transport, were high in nuclei from hypertrophic hearts, and the nuclei of sham-operated animals showed higher activities of these enzymes after incubation with supernatant from hypertrophic hearts, which stimulates the RNA transport *in vitro* from nuclei of sham-operated animals.

Transcriptional and post-transcriptional regulation and flow of genetic information from nucleus to cytoplasm in eukaryotic systems have been widely studied (Drews *et al.*, 1968; Stevenin *et al.*, 1969; Levitan & Webb, 1970; Lichtenstein & Shapot, 1976). By cell fusion and nuclear-transplantation experiments it was found that cytoplasmic factors regulate the expression of genes through feedback controls (Gurdon & Uehlinger, 1966; Harris, 1970). These feedback-control mechanisms operate not only at the transcriptional level but also at the post-transcriptional level. The availability of a cell-free system that can support nearly normal RNA processing and transport has made it possible to study the transport of RNA *in vitro*. In the present paper we report the occurrence of induced transport of RNA *in vitro* from the nuclei obtained from hypertrophic hearts compared with that from normal hearts. Schumm & Webb (1972, 1974) and Schumm *et al.* (1973) have reported that cytosol from regenerating kidney enhanced the transport capability of nuclei obtained from normal kidney, thus indicating a role for cytosol in the transport of RNA from nuclei. A similar situation is obtained in the present system of cardiac hypertrophy, and our results show the presence of some protein factors in cytosol of hypertrophic hearts that may bring about an enhanced transport activity.

Materials and methods

Female albino rats of a Wistar-derived strain were used. Animals were allowed at least 1 week to adapt

to local conditions, and animals in the weight range 150–180g were chosen for operational purposes. The animals were fed with Gold Mohur Rat Feed, from Hindustan Lever Ltd., Bangalore, India.

Chemicals

Most of the chemicals were of AnalaR grade, from BDH and E. Merck. Fine chemicals and enzymes, such as GTP (sodium salt), ATP (sodium salt), spermidine, phosphocreatine (sodium salt), creatine kinase and dithiothreitol, were obtained from Sigma. $\text{Na}_3^{32}\text{PO}_4$ was obtained from BARC, Trombay, India.

Induction of cardiac hypertrophy

Cardiac hypertrophy was induced by following the method of Rakusan & Poupa (1966) with slight modifications. The animals were anaesthetized with an intraperitoneal injection of Pentothal Sodium (40mg/kg body wt.). Positive pressure respiration was produced (respiratory rate 65/min) by using an animal respirator. Abdominal distension was prevented with a binder. The ascending aorta (systemic aorta) was partially constricted with a silver clip inserted by thoracotomy in the left fourth intercostal space, and the chest was closed rapidly. Sham operations were done in a similar way, but with no clips on the aorta. The incision was closed with surgical thread and the skin incision was closed with 12mm stainless-steel wound suture clips. The mortality during operation was about 10–12%, but no death occurred during post-operative days. The animals were killed at various time intervals after aortic constriction, and the increase in the ratio of heart weight (wet wt.) to body weight was calcu-

* To whom reprint requests should be addressed.

lated by using the formula $100 \times \text{heart weight/body weight}$. The percentage hypertrophy was calculated from the changes in the above ratio in aorta-constricted animals with respect to sham-operated controls.

Isolation of nuclei and transport of RNA in vitro

Nuclei were labelled for transport studies by injecting 0.75 mCi of ^{32}P via the intracardiac route into each animal and allowing incorporation for 1 h. The nuclei were isolated by the procedure of Muramatsu & Busch (1967), with slight modifications. After labelling, the hearts were pooled immediately and homogenized in ice-cold homogenizing buffer [0.3 M-sucrose, 0.01 M-Tris/HCl (pH 7.5), 0.01 M-NaCl, 0.001 M-MgCl₂, 0.005 M-CaCl₂, 0.1% (v/v) diethyl pyrocarbonate, 400 µg of heparin/ml, 40 µg of polyvinyl sulphate/ml], and filtered through four layers of cheesecloth. The homogenate was centrifuged at 1000 g (r_{av} 6.87 cm) for 10 min at 4°C, and the pellet was purified by pelleting through a step gradient of 2.4 M- and 1.6 M-sucrose in TEN buffer (0.01 M-Tris/HCl, 0.002 M-EDTA and 0.01 M-NaCl) at 90 000 g (r_{av} 6.4 cm) for 1 h at 4°C in a Janetzki model Vac 601 ultracentrifuge. The pellet was suspended in 1 M-sucrose containing 0.001 M-CaCl₂ and washed twice with the same buffer by centrifuging at 11 000 g (r_{av} 6.87 cm) for 10 min at 4°C to give a pellet of purified nuclei.

The incubations for transport studies were performed in a medium containing 4.5×10^6 nuclei/ml, 0.05 M-Tris/HCl (pH 7.5), 0.025 M-KCl, 0.0025 M-MgCl₂, 0.005 M-NaCl, 0.005 M-spermidine, 0.002 M-dithiothreitol, 0.002 M-ATP, 0.005 M-phosphocreatine, 35 units (1 unit = 1 µmol/min) of creatine kinase and 500 µg of yeast RNA/ml. Cytosol (100 000 g supernatant) obtained from homogenates of hypertrophic hearts or from those of sham-operated rats were dialysed against 0.01 M-Tris/HCl (pH 7.5) buffer for 18 h at 4°C with two changes, and 4.5 mg of non-diffusable material was used along with 1 ml of incubation medium. After incubation the nuclei were pelleted by centrifugation at 100 000 g (r_{av} 5.35 cm) for 1 h, and RNA was extracted from the supernatant by the method of Penman (1966). The percentage of RNA was calculated from the radioactivity (c.p.m.) in the RNA released into the supernatant per mg of cytosolic protein.

Enzyme activities

ATPase activity was assayed by the method of Tzagoloff (1970), with minor modifications. The reaction mixture contained, in a volume of 1 ml, 0.05 M-Tris/HCl, pH 8.5, 0.005 M-ATP, 0.01 M-CaCl₂, and nuclei (100–150 µg of protein) as the enzyme source. The reaction was initiated by the addition of ATP and the mixture was incubated at

30°C for 15 min. The reaction was stopped by the addition of 0.5 ml of cold 10% (w/v) trichloroacetic acid and the mixture was kept at 0°C for 30 min. The protein precipitate was centrifuged at 3020 g (r_{av} 8.25 cm) in an IEC UV-model centrifuge for 10 min at room temperature, and the phosphate content of the supernatant was measured by the method of Fiske & Subbarow (1925). The specific activity of the ATPase was measured as µmol of P released/h per mg of protein. To assay the nucleic acid-dependent ATPase activity, 25 µg of poly(A)-containing RNA was used instead of 0.01 M-CaCl₂, which is used for measuring the Ca²⁺-dependent ATPase activity.

Alkaline phosphatase activity

This was assayed by the method of Bessey *et al.* (1946). The incubation mixture contained, in a volume of 1 ml, 0.01 M-Tris/HCl, pH 9.0, 0.005 M-*p*-nitrophenyl phosphate, and nuclei (100–150 µg of protein) as the enzyme source. The mixture was incubated at 37°C for 45 min and the reaction stopped by addition of 2 ml of 0.25 M-NaOH. The suspension was mixed thoroughly and the *p*-nitrophenol released was measured by the A_{400} . The specific activity of the enzyme was calculated as µmol of nitrophenol released/h per mg of protein.

Results

Changes in heart weight during hypertrophy

The changes in the heart weight of aorta-constricted animals were compared with those for sham-operated controls at different time periods (Table 1) after surgery. The percentage hypertrophy determinations are described in the Materials and methods section. At 3 days after operation there was 17% increase in the heart-weight ratio; within 1 week the increase was 28%, and it reached 40% after 11 days of surgery. The extent of development of hypertrophy always depended on the degree of constriction of the aorta.

It has been observed in this laboratory that a decrease in the labelling of nuclear poly(A)-containing RNA at 40% hypertrophy, compared with 28% hypertrophy, was accompanied by an enhanced labelling of cytoplasmic poly(A)-containing RNA at both 28% and 40% hypertrophy (S. Meenakshi & C. Rajamanickam, unpublished work). These results prompted us to monitor the transport of RNA *in vitro* from nucleus to cytoplasm by using ^{32}P -labelled heart nuclei from sham-operated and hypertrophic rats and unlabelled cytosol. For this purpose the labelled nuclei from sham-operated and hypertrophied animals were incubated with unlabelled supernatant (post-microsomal supernatant) from sham-operated animals. The results showed that the hypertrophic-heart nuclei were capable of trans-

Table 1. *Changes in the ratio of heart weight to body weight after aortic constriction*

Percentage hypertrophy was calculated from the ratio $100 \times \text{heart weight/body weight}$ for sham-operated and aorta-constricted animals. The values of the above ratio obtained for sham-operated animals were taken as 100% and the percentage hypertrophy in aorta-constricted animals was calculated by using the formula $100 \times \text{ratio for aorta-constricted/ratio for sham}$. The values shown are means $\pm$ s.d. for three experiments. At each time point in a single experiment five animals were pooled.

	Sham-operated	Time after surgery (aortic constriction)		
		3 Days	8 Days	11 Days
$\frac{\text{Heart weight}}{\text{body weight}} \times 100$	0.29 ± 0.07	0.33 ± 0.02	0.36 ± 0.01	0.40 ± 0.07
Percentage hypertrophy	100	117	128	140

Fig. 1. *RNA transport in vitro from nucleus to cytoplasm*

Equal numbers of nuclei were incubated in transport experiments, and the radioactivity (c.p.m.) released was adjusted for constant radioactivity in the nuclei per mg of cytosolic protein added, at all points. Each point represents the mean value $\pm$ s.d. for three experiments. In all cases supernatant from sham-operated rats was used. Source of nuclei: ○, sham-operated rats; ●, hearts with 17% hypertrophy; ▲, hearts with 28% hypertrophy; ■, hearts with 40% hypertrophy.

porting more RNA into the cytosol. Maximum release of RNA (about 12%) was observed under conditions when ^{32}P -labelled nuclei from animals with 40% cardiac hypertrophy were incubated with cytosol from sham-operated animals (Fig. 1). The transported RNA was found to be intact RNA when checked by

Table 2. *Effect of heat-inactivated hypertrophic-heart supernatants on RNA transport from nuclei of sham-operated rats*

The assay conditions and calculations were as described in the text. The values are means $\pm$ s.d. for three different experiments. Incubation was for 60 min.

Source of heart cytosol	Transport (%/mg of cytosolic protein)
28% hypertrophy	10.40 ± 0.28
28% hypertrophy; heat-inactivated	2.05 ± 0.25
40% hypertrophy	14.80 ± 0.50
40% hypertrophy; heat-inactivated	3.78 ± 0.11

polyacrylamide-gel electrophoresis, and ^{32}P label was found in the rRNA peaks, which appear as the major peaks in the gel analysis.

Schumm & Webb (1972, 1974) reported an enhanced capability of nuclei obtained from normal kidney in transporting RNA *in vitro* when incubated with cytosol obtained from regenerating kidney. The enhanced capability of hypertrophic-heart nuclei in RNA transport led us to study the role of cytosol obtained from hypertrophic animals in the transport phenomenon. Fig. 2 shows that the cytosol obtained from animals at different stages of hypertrophy could induce RNA transport from nuclei obtained from sham-operated animals. This stimulatory effect was found to be more than that observed with cytosol from sham-operated animals, and the cytosol obtained from animals with 40% cardiac hypertrophy produced a maximum stimulation of the transport of RNA *in vitro*. The heat-treated heart supernatant obtained from animals with both 28% and 40% cardiac hypertrophy decreased the transport activity in nuclei of sham-operated animals to a minimum of 2% (Table 2), suggesting the probable involvement of heat-labile factors during transport. A partial enrichment

of the factors was obtained by $(\text{NH}_4)_2\text{SO}_4$ fractionation of the cytosol, and the 40–80%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction showed the maximum activity (results not shown).

Agutter *et al.* (1977) reported the involvement in

RNA transport of membrane-bound nucleoside triphosphatase, which may be stimulated by exogenous RNA. A similar involvement of alkaline phosphatase was reported by Cutler *et al.* (1974). The heart nuclei exhibited a very low activity of Mg^{2+} -dependent ATPase, whereas the Ca^{2+} -dependent activity was fairly high, and hence we measured the Ca^{2+} -dependent ATPase activity in nuclei of both normal and hypertrophic hearts. This Ca^{2+} -dependent activity could be inhibited by EGTA (see legend to Table 3).

We observed an increase in the activities of Ca^{2+} -dependent and nucleic acid-dependent ATPase in nuclei from animals with cardiac hypertrophy as compared with sham-operated controls (Table 3). The increase in alkaline phosphatase activity is comparable with the increase in ATPase activity observed in nuclei from animals with cardiac hypertrophy. The observed increase in the activities of these enzymes involved in RNA transport in nuclei from animals with cardiac hypertrophy, along with the observation of the stimulatory effect of cytosol from hypertrophic hearts on the RNA transport *in vitro* from nuclei from sham-operated animals, led us to measure these enzyme activities in the latter nuclei before and after incubation with the hypertrophic-heart supernatant. A 2-fold increase in both Ca^{2+} - and nucleic acid-dependent ATPase and alkaline phosphatase activities was observed in such nuclei after incubation with the 40–80%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction of supernatant from 40%-hypertrophic hearts (Table 4). There was a negligible increase in the enzyme activities when nuclei from sham-operated rats were incubated with the same fraction from supernatant from sham-operated rats.

Discussion

The enhanced capability of hypertrophic-heart nuclei for RNA transport *in vitro*, along with the stimulatory effect of hypertrophic-heart cytosol on RNA transport from nuclei of sham-operated animals, suggest the possibility of enhanced RNA

Fig. 2. Supernatant effect on transport of RNA
Equal numbers of prelabelled nuclei were incubated with cytosol from animals at different stages of hypertrophy, and the amounts of labelled RNA released were adjusted for constant radioactivity in the nuclei per mg of cytosolic protein added. Each point represents the mean $\pm$ S.D. for three experiments. Nuclei from sham-operated rats were used in all cases. Source of supernatant: ▼, sham-operated rats; ■, hearts with 17% hypertrophy; ○, hearts with 28% hypertrophy; ●, hearts with 40% hypertrophy.

Table 3. Ca^{2+} - and nucleic acid-dependent ATPase and alkaline phosphatase activities in the nuclei of normal and hypertrophic hearts

The values shown are means $\pm$ S.D. from three different experiments. The incubation conditions for measurement of enzyme activities and the calculations are given in the Materials and methods section. The Mg^{2+} -dependent and Ca^{2+} -dependent ATPase activities in normal nuclei in the presence and absence of EGTA were as follows: Mg^{2+} , $0.8 \mu\text{mol/h}$; Ca^{2+} , $6.98 \mu\text{mol/h}$; Mg^{2+} , +EGTA, 0; Ca^{2+} , +EGTA, $0.7 \mu\text{mol/h}$.

Source of nuclei	ATPase activity ($\mu\text{mol released/h per mg of protein}$)		Alkaline phosphatase ($\mu\text{mol of nitrophenol released/h per mg of protein}$)
	Ca^{2+} -dependent	Nucleic acid-dependent	
Sham-operated	7.42 ± 0.29	6.72 ± 0.25	3.06 ± 0.29
28% hypertrophy	9.60 ± 0.80	6.52 ± 0.85	3.72 ± 0.39
40% hypertrophy	12.56 ± 0.70	9.54 ± 0.35	4.32 ± 0.53

Table 4. *Enzyme activities in nuclei of sham-operated rats before and after incubation with 40–80%-satd.-(NH₄)SO₄ fraction of cytosol from 40%-hypertrophic hearts*

Values are the means $\pm$ S.D. for three experiments. Enzyme activities were determined as described in the Materials and methods section.

Source of cytosol 40–80%-satd.-(NH ₄)SO ₄ fractions	ATPase activity (μ mol released/h per mg of protein)		Alkaline phosphatase (μ mol of nitrophenol released/h per mg of protein)
	Ca ²⁺ -dependent	Nucleic acid-dependent	
...	7.42 $\pm$ 0.29	6.72 $\pm$ 0.25	3.06 $\pm$ 0.29
Sham	8.88 $\pm$ 0.75	7.20 $\pm$ 0.47	3.42 $\pm$ 0.32
40% hypertrophy	15.6 $\pm$ 0.61	13.08 $\pm$ 0.61	5.52 $\pm$ 1.02

transport occurring *in vivo* under hypertrophic conditions. Similar results have been obtained in drug-treated animals, where liver cytosol from drug (2-allyl-2-isopropylacetamide)-treated animals enhances the transport of RNA from normal liver nuclei (Sardana & Padmanaban, 1976; Kumar *et al.*, 1980). Murthy *et al.* (1979) observed an enhanced transport activity in normal liver nuclei when incubated with liver cytosol from tryptophan-treated animals. The above observations, along with our present results, show the presence of an active nucleo-cytoplasmic regulatory mechanism operating under a variety of conditions. The factors, present in the cytosol of animals with cardiac hypertrophy, that stimulate the transport could be protein factors, as they are heat-labile, non-diffusible and precipitable by (NH₄)₂SO₄.

Several studies on the efflux of RNA from isolated mammalian nuclei *in vitro* have indicated the ATP-dependency of the transport phenomenon (Ishikawa *et al.*, 1969, 1972; Schumm & Webb, 1972, 1974; Schumm *et al.*, 1973; Raskas & Bhaduri, 1973; Racevskis & Webb, 1974; Agutter *et al.*, 1976, 1979). Sato *et al.* (1977) observed stimulation of ribosomal ribonucleoprotein transport *in vitro* by exogenous RNA. Clawson *et al.* (1980) observed stimulation of nucleoside triphosphatase activity in calf and rat liver nuclear envelope by yeast RNA, and they suggested that the stimulatory effect of RNA may arise through a substrate effect if the nuclear-envelope enzyme requires the presence of an RNA chain. Lam & Casper (1979) and Agutter *et al.* (1976, 1977, 1979) showed a preferential phosphorylation and dephosphorylation of a particular nuclear-membrane protein of 68 000 mol.wt. during RNA transport *in vitro* from rat liver nuclei. The enhanced alkaline phosphatase and Ca²⁺- and nucleic acid-dependent ATPase activities observed with hypertrophic-heart nuclei, along with the observation of nuclei from sham-operated animals exhibiting higher activities of these enzymes after incubation with hypertrophic-heart supernatant, suggest that cytosolic factors may induce the transport through an energy-dependent

phosphorylation and dephosphorylation mechanism operating at the membrane level. Further studies are needed to characterize these factors and their exact role in the enhanced RNA transport under hypertrophic conditions.

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