Effect of Phenobarbitone on the Nucleocytoplasmic Transport of Ribonucleic Acid *in vitro*

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The transport of nucleic acids from the nucleus to the cytoplasm is a potential site for modification of normal cellular processes by drugs and hormones. In this study the effect of phenobarbitone on nucleocytoplasmic transport of ribosomes was measured in an assay system *in vitro*. The transport of radioactive ribosomes from isolated rat hepatic nuclei to unlabelled post-microsomal supernatant was measured in rats treated with 80mg of phenobarbitone/kg body wt. or saline 3h before death. With either treatment, transport was linear with time, and dependent on temperature and the presence of ATP. However, phenobarbitone treatment increased transport of ribonucleoproteins over saline-treated animals nearly twofold. The effect of phenobarbitone was mediated through the cytosol, but was not the result of altered stability of the RNA transported to the cytosol. Cycloheximide (5mg/kg body wt.) given 3.5h before death inhibited the stimulation of transport by phenobarbitone. The data indicate that phenobarbitone increased the transport of RNA by stimulating the synthesis of cytosol factors that regulate transport of RNA from the nucleus.

The high rate of degradation of RNA within the eukaryotic nucleus (Harris, 1963) suggests that nucleocytoplasmic transport of RNA is important in regulation of cell function. Shearer & McCarthy (1967) have shown that 80% of the sequences of RNA transcribed in hepatic nuclei are restricted to the nucleus and degraded. Treatment with carcinogens *in vivo* allows sequences usually restricted to the nucleus to appear in the cytoplasm (Shearer, 1974). Other data indicate (Cooper, 1969; Cooper & Gibson, 1971) that in resting lymphocytes 50% of the ribosomal subunits synthesized are degraded before reaching the cytoplasm. However, Cooper (1969) and Cooper & Gibson (1971) have shown that if resting lymphocytes are stimulated to proliferate with phytohaemagglutinin, there is an increase in survival of 18S and 28S RNA, and over 90% of the rRNA transcribed is recovered in the cytoplasm. The phytohaemagglutinin effect is prevented by previous treatment with cycloheximide. Recent studies *in vitro* by Webb and co-workers (Yu *et al.*, 1972; Schumm & Webb, 1972) have shown that the transport of both ribosomal and presumptive mRNA from isolated hepatic nuclei is an ATP- and temperature-dependent process. This transport requires protein factors (Schumm *et al.*, 1973a; Racevskis & Webb, 1974) present in the cytosol.

In the present paper, the possibility that phenobarbitone influences the post-transcriptional control

of the nucleocytoplasmic transport of ribosomes has been investigated. Shortly after the administration of a single dose of phenobarbitone *in vivo* there is an increased incorporation of radioactive precursors into cytoplasmic RNA (Cohen & Ruddon, 1970; McCauley & Couri, 1971; Smith *et al.*, 1972). This RNA appears principally in ribosomes bound to the endoplasmic reticulum (McCauley & Couri, 1971), and occurs before proliferation of the smooth endoplasmic reticulum (Orrenius *et al.*, 1965; Staubli *et al.*, 1969).

We have used the system described by Yu *et al.* (1972) to assay *in vitro* the effect of phenobarbitone on RNA transport. Our experiments indicate that 3h after a single dose of 80mg of phenobarbitone/kg body wt., the rate of transport of ribosomes from rat liver nuclei is increased nearly twofold. Further, it appears that this effect is mediated by cycloheximide-sensitive factors present in the liver cytosol.

A preliminary report of this work has been presented at the Meeting of the American Society for Pharmacology and Experimental Therapeutics, Montreal, Canada (Hazan & McCauley, 1974).

Materials and Methods

Treatment of animals

Male Sprague-Dawley rats (150-200g) were starved for approx. 20h before use. All drug and isotope injections were given intraperitoneally, and

control animals were given an equal volume of saline (0.9% NaCl). Phenobarbitone (80mg/kg body wt.) was administered 3h before death; when indicated cycloheximide (5mg/kg body wt.) was administered 3.5h before death.

Tissue preparation

In all instances animals were killed by decapitation, and their livers were perfused *in situ* through the portal vein with about 40ml of ice-cold 0.9% NaCl. Livers were excised, and passed through a Harvard Tissue Press, before homogenization.

Nuclei were prepared from livers of animals that had been injected with 100 μ Ci of [^{14} C]orotate/kg body wt. (55mCi/mmol, New England Nuclear Corp., Boston, MA, U.S.A.) 3h before death. Each liver was homogenized in 120ml of 2.4M-sucrose/3.3mM-CaCl₂ with a Teflon/glass tissue grinder. The homogenate was filtered through 12 layers of cheesecloth, and then centrifuged for 1h at 4°C in a Spinco 30 rotor at 30000rev./min. The nuclear pellet was resuspended in 10ml of 0.25M-sucrose/3.3mM-CaCl₂ and centrifuged at 2300rev./min for 10min in a Sorvall SS34 rotor. The nuclei were washed once more, and then finally resuspended by gentle hand homogenization in 1.5ml of 0.25M-sucrose/3.3mM-CaCl₂. This procedure is a modification of the method described by Muramatsu *et al.* (1966).

The cytosol used in incubations was prepared from animals that had not been treated with [^{14} C]orotate. Usually two livers from similarly treated animals were pooled and homogenized in 15ml of 0.25M-sucrose/40mM-Tris/2mM-EDTA, pH7.4. The homogenate was centrifuged at 9500rev./min in a Sorvall SS34 rotor for 10min. The supernatant was then centrifuged at 40000rev./min in a Spinco 40 rotor for 1h. This supernatant was dialysed for 3h against 100vol. of water and kept frozen (-20°C) overnight before use.

Assay of RNA transport

The transport of RNA from isolated nuclei was measured by a modification of the method described by Yu *et al.* (1972). Duplicate samples of radioactive nuclei (about 2mg of DNA/ml) and cytosol (10mg of protein/ml) were incubated at 35°C in a medium containing 50mM-Tris/HCl buffer (pH7.8), 250mM-sucrose, 2.5mM-MgCl₂, 25mM-KCl, 0.5mM-CaCl₂, 0.3mM-MnCl₂, 2.5mM-Na₂HPO₄, 5mM-NaCl, 5mM-spermidine, 1mg of yeast RNA/ml, 2.5mM-dithiothreitol and an ATP-regenerating system consisting of 2.0mM-ATP, 2.5mM-phosphoenolpyruvate and 6.4 units of pyruvate kinase/ml. Sufficient phosphoenolpyruvate was added at 5min intervals to keep the final concentration at 2.5mM. In all instances, similar samples were incubated in ice as an index of background radioactivity. The incubations were

terminated by immersing the incubation vessels in ice and, except where indicated, all subsequent operations were at 4°C. After incubation, the nuclei were removed by centrifugation at 3000rev./min for 10min in an International Equipment Company model V centrifuge. HClO₄ was added to the supernatant to a final concentration of 7% (v/v) and centrifuged. The precipitate was washed twice with 5% (v/v) HClO₄, and hydrolysed overnight at 35°C in 0.5M-NaOH. A sample was neutralized with 4M-HCl before measurement of radioactivity. These samples usually contained 600–1000c.p.m. The perchloric acid supernatant and washes were combined and neutralized with 10M-KOH. The KClO₄ was removed by centrifugation and a sample was assayed for radioactivity. The perchloric acid precipitate was considered to be transported RNA. The acid-soluble counts represent unincorporated orotate and oligonucleotides. Both fractions were assayed as a routine. In all Figures and Tables data represent the difference between 0° and 35°C incubations, and are expressed as percentage of radioactivity initially present in nuclei.

Characterization of transported nucleoproteins

Rats were injected with 250 μ Ci of [^{14} C]orotate/kg body wt. for 2h before death and nuclei were prepared as described above. The incubations were carried out by using supernatants from either saline- or phenobarbitone-treated animals. A 2ml sample of the incubation medium, freed of nuclei, adjusted to 50mM-EDTA and containing 0.5mg of ribosomes (Bloemendal *et al.*, 1967) was layered on to a continuous 10–25% sucrose gradient, which had been prepared in 30mM-Tris/HCl (pH7.6)/25mM-NaCl. The gradients were centrifuged for 16h at 25000rev./min in an SW 27 rotor. Fractions (approx. 1ml) were collected, and E_{260} and radioactivity were measured.

Analytical methods

DNA and protein concentrations were measured by the diphenylamine (Burton, 1956) and biuret (Gornall *et al.*, 1949) assays respectively. Radioactivity was measured in Aquasol (New England Nuclear) by liquid-scintillation spectroscopy (Beckman LS 100C) at about 85% efficiency for ^{14} C. Alkaline ribonuclease was measured by the method of Tuve & Anfinsen (1960).

Results

As can be seen from Fig. 1, when rat liver nuclei and cytosol were incubated as described in the Materials and Methods section, radioactive RNA was transported at a constant rate over a 40min period. The transport of radioactivity is an active process since when incubations were performed at

Fig. 1. Release of radioactivity from liver nuclei of control and phenobarbitone-treated rats

Nuclei (2mg of DNA/ml) from rats treated with 100 μ Ci of [^{14} C]orotate/kg body wt. and either saline or 80mg of phenobarbitone/kg body wt. 3h before death were incubated at 35°C with 10mg of cytosol protein from rats treated with saline or phenobarbitone for 3h/ml. Incubation and assay conditions were as described in the Materials and Methods section. The main Figure shows the radioactivity in acid-precipitable RNA. The inset Figure shows the radioactivity in the acid-soluble fraction. ●, Saline-treated; ▲, phenobarbitone-treated; ■, saline-and-phenobarbitone-treated, but assayed in the absence of ATP. Numbers of determinations are given in parentheses; bars indicate $\pm$ S.E.M.

Table 1. Effect of actinomycin D on RNA transport *in vitro*

Nuclei (2mg of DNA/ml) and cytosols (10mg of protein/ml) from rats treated for 3h with phenobarbitone (80mg/kg body wt.) or saline were incubated for 40min and assayed as described in the Materials and Methods section, and in the legend to Fig. 1. When indicated, 10 μ g of actinomycin D/ml was included in the incubation. In both this and subsequent Tables the results are expressed as means $\pm$ S.E.M., with the numbers of determinations in parentheses.

Treatment of cell components		Percentage of nuclear radioactivity transported in 40min
Nuclei	Cytosol	
Control	Control	1.68 $\pm$ 0.17 (4)
Control	Control+	1.87 $\pm$ 0.15 (5)
	actinomycin D	
Phenobarbitone	Phenobarbitone	3.81 $\pm$ 0.56* (3)
Phenobarbitone	Phenobarbitone+	3.38 $\pm$ 0.28* (5)
	actinomycin D	

* Significantly different from control nuclei and control cytosol, $P < 0.01$.

0°C or in the absence of ATP only a constant background of less than 1% of the nuclear radioactivity was observed. Acid-soluble radioactivity also increased with time, but was not entirely ATP-dependent (Fig. 1, inset).

When nuclei and cytosol from rats treated for 3h with 80mg of phenobarbitone/kg body wt. were incubated under the same conditions, RNA accumulation in the cytosol was approximately linear with time and entirely ATP-dependent; however, the rate of RNA accumulation was greater. For example, in incubations with phenobarbitone-treated nuclei and cytosol, 2.60 $\pm$ 0.18% of the nuclear radioactivity was transported as compared with 1.44 $\pm$ 0.34% for controls. There were no differences in the amount of extranuclear acid-soluble radioactivity. These differences in apparent RNA transport were not due to differences in RNA synthesis *in vivo*, since phenobarbitone treatment has no effect on the labelling of the nuclear fractions (17200 $\pm$ 2800 and 17300 $\pm$ 2400 c.p.m./mg of DNA for control and phenobarbitone-treated nuclei respectively). Further, the presence of 10 μ g of actinomycin D/ml (Table 1) had no effect on either control or phenobarbitone incubations, indicating that continuing RNA synthesis is not required for RNA transport *in vitro*.

Webb and co-workers (Yu *et al.*, 1972; Schumm *et al.*, 1973a,b; Racevskis & Webb, 1974) have suggested that certain unidentified proteins in the cytosol mediate RNA transport from isolated nuclei. Experiments shown in Table 2 indicate that phenobarbitone may affect RNA transport through factors present in the cytosol. For example, when control nuclei were incubated in the presence of phenobarbitone-treated cytosol, they were almost as active as phenobarbitone-treated nuclei incubated in phenobarbitone-treated cytosol. On the other hand, phenobarbitone-treated nuclei in control cytosol were only as active as the control nuclei in their own cytosol. Apparently the source of the cytosol and not the nuclei determines the rate of RNA transport. Also in Table 2 addition of 0.1 mM-phenobarbitone to control incubations did not increase the rate of RNA transport. These data suggest that phenobarbitone does not alter transport through a direct interaction with either the nuclear membranes or the cytosol.

It seemed possible that instead of an increase in the rate of transport, the greater accumulation of RNA in phenobarbitone incubations might be the result of greater stability of the newly transported RNA in the cytosol. In Table 3, control and phenobarbitone-treated cytosol were incubated with control nuclei for 40min at 35°C. The nuclei were removed by centrifugation, and the amount of radioactive RNA was determined in one sample of the cytosol. Another sample was incubated for an additional 40min, and radioactivity remaining in

Table 2. *Effect of phenobarbitone treatment on RNA-transport activity of the hepatic cytosol*

Nuclei (2mg of DNA/ml) and cytosol (10mg of protein/ml) from rats treated for 3h with phenobarbitone (80mg/kg body wt.) or saline were incubated for 40min as indicated. In one instance 0.1mM-phenobarbitone was added to the incubation mixture; otherwise incubation and assay conditions were as described in the Materials and Methods section, and in the legend to Fig. 1.

Treatment of cell components		Percentage of nuclear radioactivity transported in 40min
Nuclei	Cytosol	
Control	Control	2.37 ± 0.19 (15)
Phenobarbitone	Phenobarbitone	4.01 ± 0.48* (6)
Control	Phenobarbitone	3.44 ± 0.19* (11)
Phenobarbitone	Control	2.86 ± 0.43 (5)
Control	Control+0.1M-phenobarbitone	2.03 ± 0.25 (4)

* Significantly different from control nuclei and control cytosol, $P < 0.01$.

Table 3. *Stability of transported RNA in vitro*

Nuclei (2mg of DNA/ml) from saline-treated rats and cytosols (10mg of protein/ml) from rats treated for 3h with phenobarbitone (80mg/kg body wt.) or saline were incubated for 40min as described in the Materials and Methods section, and in the legend to Fig. 1. The nuclei were removed by centrifugation, and the post-nuclear supernatant was divided into two fractions. RNA transport was assayed immediately in one fraction (40min), and after another 40min incubation at 35°C (80min) in the other.

Treatment of cytosol	Time of incubation (min)	Percentage of nuclear radioactivity transported
Control	40	2.55 ± 0.34 (6)
Control	80	2.34 ± 0.53 (6)
Phenobarbitone	40	4.17 ± 0.53* (6)
Phenobarbitone	80	3.83 ± 0.39† (6)

* Significantly different from control at 40min, $P < 0.05$.

† Significantly different from control at 80min, $P < 0.05$.

RNA was measured. The amount of RNA present in either control or phenobarbitone-treated cytosol was virtually unchanged by the additional incubation, indicating that newly transported RNA was equally stable in either cytosol. Consistent with these data is the observation that alkaline ribonuclease activity in control and phenobarbitone-treated cytosols was identical (0.65 μ g of RNA hydrolysed/30min per mg of protein).

Ribosomal RNA is released *in vivo* from the nucleus into the cytoplasm as 40S and 60S ribonucleoproteins (Girard *et al.*, 1965). The sucrose-

density-gradient analysis in Fig. 2 indicates a substantial portion of the radioactivity is released from the nuclei as ribonucleoprotein particles. In three experiments an average of 58.8% of the acid-precipitable radioactivity in both control and phenobarbitone experiments was recovered as 40S and 60S particles. In the absence of ATP no rapidly sedimenting particles were released. In incubations with phenobarbitone-treated cytosol and control nuclei, the release of 60S ribonucleoprotein particles was twice as great as controls. Phenobarbitone had less effect on the release of 40S particles, although in some experiments a shoulder of slower sedimenting radioactivity was associated with the 40S peak. It appears that under the conditions of this assay

Fig. 2. *Sucrose-density-gradient analysis of the ribonucleoproteins transported*

Nuclei (2mg of DNA/ml) from rats treated with 250 μ Ci of [^{14} C]orotate 2h before death were incubated at 35°C for 30min with 10mg of cytosol protein/ml from rats treated with saline or 80mg of phenobarbitone/kg body wt. for 3h. After removal of the nuclei by centrifugation, the supernatant was brought to 50mM-EDTA and 0.5mg of rat liver ribosomes was added. The mixture was layered on a continuous 10–25% sucrose gradient and centrifuged for 16h at 25000 rev./min. Fractions (1 ml) were collected, and E_{260} and radioactivity measured. ●, Saline-treated; ▲, phenobarbitone-treated; ■, saline-treated, but assayed in the absence of ATP. The 40S and 60S standards are indicated above.

Table 4. *Effect of cycloheximide treatment on RNA-transport activity of the hepatic cytosol*

Nuclei (2mg of DNA/ml) from rats treated for 3h with phenobarbitone (80mg/kg body wt.) and saline were incubated with cytosols from similarly treated rats and from rats treated with 5mg of cycloheximide/kg body wt. 30min before phenobarbitone or saline treatment. Otherwise incubation and assay conditions were as described in the Materials and Methods section, and in the legend to Fig. 1.

Treatment of cell components		Percentage of nuclear radioactivity transported in 40 min
Nuclei	Cytosol	
Control	Control	2.00 ± 0.25 (6)
Phenobarbitone	Phenobarbitone	3.38 ± 0.37* (6)
Control	Control + cycloheximide	2.17 ± 0.39 (6)
Phenobarbitone	Phenobarbitone + cycloheximide	2.56 ± 0.13 (6)

* Significantly different from control nuclei and control cytosol, $P < 0.02$.

radioactivity is transported as ribonucleoproteins similar in size to ribosomal subunits isolated from rat liver.

Collectively, the data in Figs. 1 and 2, and Tables 1, 2 and 3 indicate that in our assay phenobarbitone treatment enhances the nucleocytoplasmic transport of ribosomal RNA as ribonucleoproteins. This enhanced transport is independent of alterations in RNA synthesis or degradation, and is mediated by factors in the cytosol.

The possibility that treatment with phenobarbitone increases the synthesis *de novo* of protein transport factors in the cytosol was investigated. In Table 4, the cytosols from animals that had received 5mg of cycloheximide/kg body wt. 30min before either phenobarbitone or saline were compared with cytosols from similar animals that did not receive cycloheximide. Cycloheximide pretreatment had no effect on the transport activity of control cytosols, but the same treatment lowered the activity of phenobarbitone-treated cytosols to control values. These observations suggest that phenobarbitone induced the synthesis of factors involved in the nucleocytoplasmic transport of RNA.

Discussion

Phenobarbitone increases the appearance of ribosomal RNA in rat liver cytoplasm *in vivo* within 24h after a single treatment (Cohen & Ruddon, 1970; McCauley & Couri, 1971; Smith *et al.*, 1972). It has been suggested that phenobarbitone increases the synthesis of rRNA (Gelboin *et al.*, 1967; Wold & Steele, 1972) and that the stability of RNA in

cytoplasm of phenobarbitone-treated rats is increased (Cohen & Ruddon, 1970). Smith *et al.* (1972) have reported that the rate of synthesis *in vivo* of nucleolar precursors of rRNA was not substantially increased, but that during incubations *in vitro* the nucleolar 45S RNA was more stable in the nuclei of phenobarbitone-treated rats. In a subsequent study Smith *et al.* (1974), have shown that phenobarbitone treatment did not affect the activity of either nuclear alkaline ribonuclease or nucleolar RNA polymerase, but did increase methylation of nucleolar 45S RNA. It was concluded that phenobarbitone may enhance the nuclear stability of ribosomal precursors by increasing the methylation of this RNA species. Presumably these more stable precursors would lead to more efficient processing and consequent transport into the cytoplasm.

We have used an assay *in vitro* described by Yu *et al.* (1972) to measure directly the effect of phenobarbitone on the nucleocytoplasmic transport of ribosomes. Data presented here indicate that after only 3h of treatment, phenobarbitone increases the rate of RNA transport as ribonucleoproteins from isolated nuclei. Interestingly, this effect is mediated through factors present in the cytosol, whereas the nuclei seem to have little influence. Therefore it would seem that the effects that phenobarbitone may have on the stability of nuclear RNA *in vivo* are not essential for increased RNA transport.

The data presented here imply that phenobarbitone treatment enhances the nucleocytoplasmic transport of RNA; however, it could be argued that the apparently low rates of RNA transport (about 2-4% of the nuclear radioactivity/h) are not biologically significant. Stewart & Farber (1968) have shown that in rat liver, rRNA continues to appear in the cytoplasm for about 14h after labelling *in vivo*, whereas tRNA continues to appear for more than 20h. Assuming that a large percentage of the RNA is degraded in the nucleus before transport (Harris, 1963; Cooper, 1969; Cooper & Gibson, 1971; Shearer & McCarthy, 1967), it is possible to conclude that the rates observed in the cell-free system are comparable with the expected rates *in vivo*.

The process by which phenobarbitone increases RNA transport is not clear. Apparently the barbiturate does not exert its effect by binding directly to either nuclear or cytoplasmic components since addition of the drug *in vitro* has no effect. It seems more likely that phenobarbitone may increase transport by inducing the synthesis of regulatory proteins found in the cytosol since previous administration of cycloheximide prevents the increase in transport usually observed with the cytosol of phenobarbitone-treated rats. In this connexion it is interesting that cycloheximide

treatment *in vivo* also prevents the phenobarbitone-stimulated appearance of RNA in the rough endoplasmic reticulum (McCauley & Couri, 1971). Of course, effects of cycloheximide not related to inhibition of protein synthesis cannot be excluded.

Whatever the mechanism, phenobarbitone has been shown to affect the rate of transport of ribosomal subunits from the nucleus by increasing the activity of cytosol factors. Racevskis & Webb (1974) have described protein factors in the cytosol of rat liver that affect the rate of transport of RNA. Schumm & Webb (1972) and Schumm *et al.* (1973b) have shown that the rate of transport and the nature of the RNA transported depend on whether the source of the cytosol is normal, regenerating or neoplastic hepatic tissue. Webb and co-workers (McNamara *et al.*, 1975) have shown that cytosol factors may also be important in regulating RNA synthesis. It is possible that cytosol factors may be important regulators of nuclear activity, and that phenobarbitone may be only one example of drugs that interfere with their normal function. In this regard the effect of chemical carcinogens on cytoplasmic control of RNA transport *in vitro* might shed light on their effects *in vivo* on nucleocytoplasmic movement of RNA (Shearer, 1974).

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