# THE RELATIONSHIP BETWEEN POLYRIBONUCLEOTIDE BINDING AND THE PHOSPHORYLATION AND DEPHOSPHORYLATION OF NUCLEAR ENVELOPE PROTEIN

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## 1. Introduction

A nucleoside triphosphatase (EC 3.6.1.15) present in some mammalian nuclear envelopes appears to supply the energy for the nucleo-cytoplasmic transport of some RNA species [1–8]. A single polypeptide, as revealed by sodium dodecylsulphate (SDS)–polyacrylamide gel electrophoresis, becomes phosphorylated during the hydrolysis of ATP by this enzyme [9,10], and some workers have concluded that the nuclear envelopes contain both a protein kinase [10,11] and a phosphoprotein phosphohydrolase [12] which together constitute the 'nucleoside triphosphatase [9]. In isolated liver nuclear envelopes, the overall nucleoside triphosphatase activity is stimulated by certain exogenous polyribonucleotides, notably by poly(G) and to a lesser extent by poly(A) [13]. This stimulation, observed only when the polyribonucleotide has a highly organized tertiary structure [14], may be relevant to the role of the enzyme in nucleo-cytoplasmic RNA transport [3]. However, the exact relationship between nuclear envelope protein phosphorylation and dephosphorylation, and the binding and release of RNA to and from the transport system, remains obscure. The object of this investigation was to elucidate this relationship. The results suggest that ATP binding to the pore-lamina stimulates the release of bound polyribonucleotides. Dephosphorylation of the phosphorylated nuclear envelope protein, which is the rate-limiting step in the process, is stimulated by exogenous poly(G). These results are consistent with the scheme proposed in [5] (cf. [15]).

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## 2. Materials and methods

### 2.1. *Isolation of nuclear envelopes and pore-laminae*

Nuclear envelopes were obtained from sheep liver by a modification [16] of the method in [17]. This method gives material with a high nucleoside triphosphatase activity [9,18]. The isolated envelopes were treated with RNAase (Sigma) to remove endogenous polyribonucleotides [13]. Pore-laminae were isolated as in [15,19].

### 2.2. *$^{32}$P-Labelling of nuclear envelope protein*

Nuclear envelopes (~50 μg protein) were incubated with 8 μmol [γ-$^{32}$P]ATP (20 Ci/mmol) for 0–30 min at 20°C in 1 ml 50 mM Tris–HCl (pH 8.0), 1 mM $MgCl_2$ 10 mM NaF ± 100 μg poly(G) (Sigma) [13,14]. The reaction was terminated by the addition of cold unlabelled 10 mM $MgATP^{2-}$ + 20 μg quercetin (Sigma Chemical Co.) ml: quercetin at this concentration completely inhibits phosphorylation [9]. The nuclear envelopes were recovered by centrifugation at 50 000 × *g* for 15 min, washed with 50 mM Tris–HCl, 1 mM $MgCl_2$, 10 mM NaF, resuspended in 200 μl water, and counted at 25% efficiency in 5 ml (7:3) toluene:Triton X-100 containing 0.4% 2,5-diphenyloxazole (PPO) and 0.003% 1,4-bis-(5-phenyloxazole-2-yl)benzene-(POPOP), using a Packard Tri-Carb Liquid Scintillation Counter.

### 2.3. *Dephosphorylation of nuclear envelope protein*

Nuclear envelopes were phosphorylated for 30 min under the above conditions. The reaction was terminated by the addition of cold unlabelled 10 mM $MgATP^{2-}$. The washed envelopes were resuspended in 1 ml 50 mM histidine–imidazole, 1 mM $MgCl_2$ (pH 7.3) ± 100 μg poly(G) [12], incubated for 0–60

min at 20°C, recovered by centrifugation at 50 000 × *g* for 15 min, and counted as before.

2.4. *Poly(A) binding*

Poly(A) was used for these experiments because radioactively labelled poly(G) is not commercially available. Nuclear envelopes or pore-laminae (~50 μg protein) were incubated with 0.05–0.5 nmol poly([8-$^3$H]A) (Amersham, 15 Ci/mmol) for 10 min at 20°C in 1 ml 50 mM Tris–HCl, 1 mM $MgCl_2$ (pH 8.0) [13]. The suspension was layered over 30% (w/v, sucrose) in 50 mM Tris–HCl, 1 mM $MgCl_2$ (pH 8.0) and centrifuged at 50 000 × *g* for 30 min. The pellets were resuspended in 200 μl water, mixed with 5 ml 99.5% toluene, 0.45% PPO, 0.05% POPOP, and counted at 15% efficiency in a Packard Tri-Carb Liquid Scintillation counter. Aliquots (200 μl) of supernatant were counted similarly. In some experiments, the nuclear envelopes or pore-laminae were phosphorylated for 30 min with unlabelled ATP, as in section 2.2, before incubation with poly([8-$^3$H]A).

2.5. *Chemical assays*

Protein was determined by a modification [20] of the method in [21], DNA by the method in [22] RNA by the method in [23], and phospholipid as in [16].

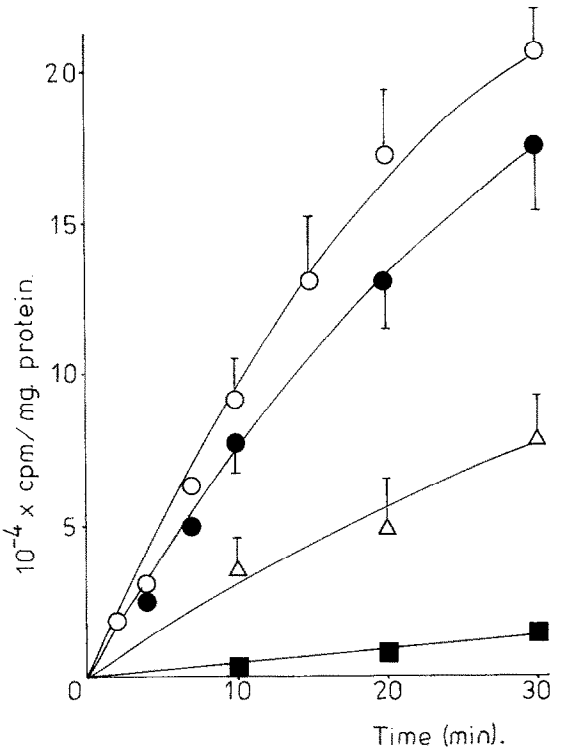


Fig.1. Nuclear envelope protein phosphorylation. Time course of incorporation of $^{32}$P in the presence of poly(G) (•) and quercetin (■), and in the absence of $F^-$ (△); Controls (○). Results are means ± SEM of determinations on 4 preparations.

## 3. Results and discussion

3.1. *Protein phosphorylation*

The nuclear envelopes were substantially free of contamination by other subcellular components, and had the composition 72 ± 5% protein, 23 ± 2% phospholipid, 4 ± 1% DNA, and <1% RNA [9,18,24]. ATP-dependent phosphorylation of the protein was half-complete in 16 ± 3 min at 20°C, and was inhibited to some extent ($p < 0.05$ by analysis of variance) by 100 μg poly(G)/ml (fig.1). In the absence of $F^-$, the phosphate incorporated into the envelopes was decreased by ~70%. This is consistent with the view that $F^-$ inhibits the phosphoprotein phosphohydrolase activity of the envelopes [12]. In the presence of 20 μg quercetin/ml, very little phosphate incorporation occurred [9]. Less than 10% of the radioactivity incorporated in a 30 min incubation in either the presence or absence of $F^-$ was solubilized by 2% (v/v) Triton X-100 [19]; this is consitent with the conclusion that the polypeptide preferentially phosphorylated by ATP is a pore-lamina component [9,10].

3.2. *Dephosphorylation*

At pH 7.3, 20°C, dephosphorylation was half-complete in 54 ± 9 min in the absence of poly(G), and in 28 ± 6 min in the presence of 100 μg poly(G)/ml (fig.2). The reaction was almost completely inhibited by $F^-$ [12]. Thus, nuclear envelope protein dephosphorylation is markedly slower than phosphorylation. Also, the rate of dephosphorylation is about doubled by saturating concentrations of poly(G), as is the nucleoside triphosphatase reaction rate [14]. A phosphoprotein phosphohydrolase in nuclear envelopes [12] hydrolysed exogenous substrates, such as phosvitin, at pH 6.7. In our experience, the optimum pH for this dephosphorylation of phosvitin is ~6.0, while that for the dephosphorylation of the endogenous phosphoprotein described here is 7.3. This result suggests that nuclear envelope preparations contain at least two phosphoprotein phosphohydrolase activities, only one of which appears on the basis of

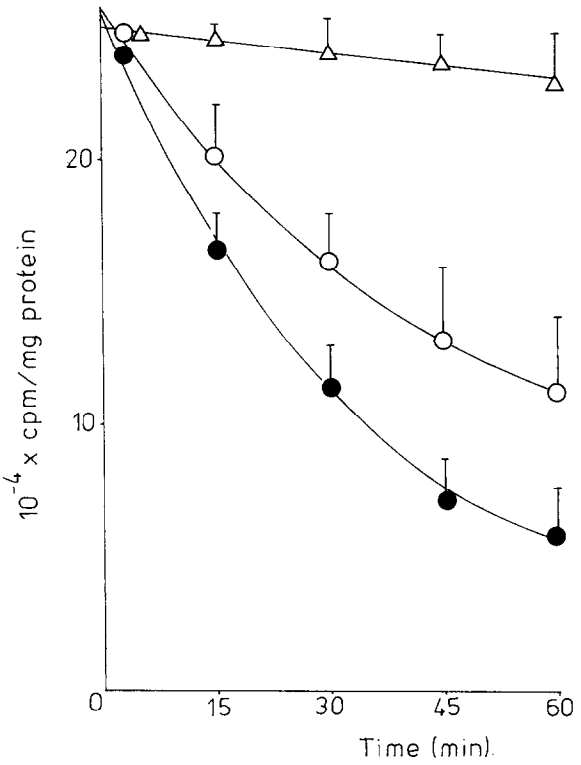


Fig.2. Dephosphorylation of nuclear envelope proteins. Time course of removal of $^{32}P$ from nuclear envelopes in the presence of poly(G) (•) and $F^-$ (△); Controls (○). Results are means ± SEM of determinations on 4 preparations.

this investigation to be involved with the RNA transport system. This view is supported by the observations that:

(i) Phosvitin (100 or 500 μg/ml) does not compete with the dephosphorylation described in fig.2;

(ii) Phosvitin hydrolysis is only ~50% inhibited by 10 mM $F^-$.

### 3.3. *Polyribonucleotide binding to the pore-lamina*

No clear binding data could be obtained from whole nuclear envelopes, but linear Scatchard plots (fig.3) were obtained using pore-laminae. This may indicate that nuclear envelopes contain a heterogeneous population of RNA binding sites, but pore-laminae contain predominantly a single class. The affinity of the pore-lamina for poly(A) is markedly increased by phosphorylation of the protein, but phosphorylation does not appear to alter the total number of binding sites. The dissociation constant

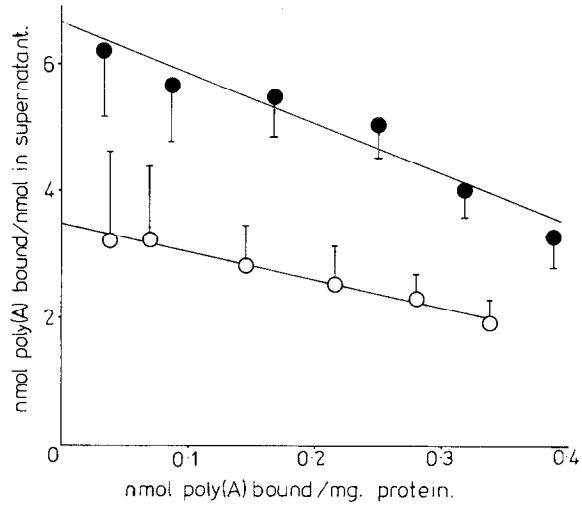


Fig.3. Binding of poly(A) to pore-laminae. Scatchard plot showing binding of poly(A) to phosphorylated (•) and unphosphorylated (○) pore-laminae. Results are means ± SEM of determinations on 3 preparations. Half-maximal saturation occurs with 0.12 nmol poly(A)/mg protein in phosphorylated, 0.25 nmol poly(A)/mg protein in unphosphorylated, pore-laminae, under the conditions used (see section 2.4); 1 nmol = ~100 μg.

Table 1
Release of bound poly([8-$^3$H]A) from pore-laminae

| Buffer supplement | cpm × $10^{-5}$/mg supernatant | Pore-lamina protein in pore-lamina pellet |
|---|---|---|
| – (Control) | 0.8 ± 0.3 | 8.2 ± 0.5 |
| 1 mM ATP | 6.6 ± 0.7 | 2.7 ± 0.4 |
| 1 mM [β,γ-methylene]-ATP | 5.7 ± 0.5 | 3.2 ± 0.4 |
| 1 mM EDTA | 1.5 ± 0.5 | 7.7 ± 0.6 |

Pore-laminae were recovered after incubation with 0.5 nmol poly(A) (see section 2.4 of text), incubated for 10 min at 20°C in 50 mM Tris–HCl, 1 mM $MgCl_2$ (pH 8.0) then recovered again as in section 2. Results are means ± 1 SEM of 3 determinations

for the phosphorylated system (0.12 nmol/mg protein) is consistent with the value for half-maximal stimulation of the nucleoside triphosphatase by poly(G), obtained from kinetic studies [14]. Together with the observed stimulation of dephosphorylation by poly(G) (see section 3.2), these results suggest that RNA binds preferentially to the phosphorylated protein and stimulates the removal of the phosphate [5].

Table 1 summarizes results showing that ATP and [β,γ-methylene]-ATP (Boehringer) stimulated the release of bound poly([$^3$H]A) from the pore-lamina. This effect was not simulated by EDTA. Since the efficacy of [β,γ-methylene]-ATP in releasing the label was comparable with that of ATP itself, this process is probably independent of ATP hydrolysis. If these findings are relevant to the behaviour of the nucleocytoplasmic RNA transport system in vivo, then it seems likely that the binding of ATP or an analogue to a nuclear envelope structure (probably in the pore-lamina) is responsible for the release of bound RNA into the cytoplasm. This is consistent with the conclusions in [15]. However, the binding of RNA itself to this structure may be dependent on prior nucleoside triphosphate-dependent phosphorylation of a pore-lamina polypeptide [5,9,10], and the concomitant dephosphorylation appears to be the rate-limiting step of the whole process.

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