# THE IN VITRO STIMULATION OF THE INFLUENZA TRANSCRIPTASE BY RIBOSOMES

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## 1. Introduction

The activity of the influenza transcriptase associated with infected cells can be assayed under two different conditions: either with $Mg^{2+}$ ions at low ionic strength or with $Mn^{2+}$ ions at high ionic strength [1–5]. The activity of the influenza virion-associated transcriptase can be assayed in the presence of $Mn^{2+}$ at high ionic strength, whereas the activity is very poor or absent in the presence of $Mg^{2+}$ ions at low ionic strength [4–6]. The purpose of this investigation was to find the basis for the difference in transcriptase activity between the virion and the infected cells. An obvious explanation would be that there are two different enzymes in the infected cells and only one is incorporated into the virion. However, this does not seem likely since the two cell-associated transcriptase activities cannot be separated physically [5]. The idea of looking for a cellular factor came from the observation that in infected cells the transcriptase is found associated with a heavy complex which sediments together with monosomes in a sucrose gradient [5]. Therefore, an assay of the cell-associated transcriptase always contains ribosomes. To attempt to reconstitute the cellular environment for the assay of the virion-associated transcriptase, ribosomes were added to the viral system. It was found that ribosomes stimulated the virion-associated transcriptase in the presence of $Mg^{2+}$ ions and low ionic strength, eliminating the difference in transcriptase activity between the virion and the infected cells.

## 2. Materials and methods

### 2.1. *Purification of the virus*

Influenza $A_0$/NWS and $A_2$ Singapore 1/57 were propagated in eggs or in cultures as previously described [5]. The virus was purified by precipitation with 7% polyethyleneglycol and 2.3% NaCl, or with 50% ammonium sulfate, differential centrifugation on sucrose gradients, and banding in a tartrate gradient [2].

### 2.2. *Disruption of the virions*

The virions were disrupted in Tris–HCl, pH 8, Triton N101, sodium deoxycholate, urea and 2-mercaptoethanol at final concentrations of 8 mM, 0.3%, 200 mM and 10 mM, respectively, for 2 min at 20°C. This treatment liberates an active transcriptase complex [8,9]. 10 or 20 μl of this mixture containing 40–70 μg viral protein were used in a transcriptase assay.

### 2.3. *Transcriptase assays*

The transcriptase assays were done under the optimal conditions described in [4]. The reaction mixture contained the following components: 80 mM Tris–HCl, pH 8.0, 10 mM 2-mercaptoethanol, 0.8 mM ATP, 0.4 mM GTP, 0.4 mM UTP, 0.2 mM [$^3$H]CTP (10 mCi/mmol), 2 mM $MgCl_2$ or $MnCl_2$, 30 mM KCl ($Mg^{2+}$ assay) or 200 mM KCl ($Mn^{2+}$ assay) and 40–70 μg of disrupted virions. The mixture, in a volume of 0.4 ml, was incubated at 37°C for 60 min and the reaction was terminated by the addition of 5 ml 5% TCA and 0.5 ml 0.1 M $Na_4P_2O_7$. The precipitates were collected on Whatman GF/C glass fiber filters. The filters were dried and the radioactivity measured in a toluene-based scintillator solution.

### 2.4. *Preparation of ribosomes*

HeLa cells or calf kidney in primary cultures were


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homogenized in hypotonic buffer containing 30 mM KCl, 10 mM Tris–HCl, pH 8.0, 1 mM $MgCl_2$. The homogenate was centrifuged at 15 000 *g* for 20 min. The supernatant was adjusted to 1% deoxycholate and then centrifuged at 130 000 *g* for 90 min at 4°C. The pellet was resuspended in the hypotonic buffer described above. To wash ribosomes with KCl, solid KCl was added slowly to the suspension of ribosomes to a final concentration of 1 M, the mixture was left at 0°C for 1 h, and then centrifuged as described above. The pellet was resuspended in 8 mM Tris–HCl, pH 8.0, containing 30 mM KCl and dialyzed against the same buffer.

## 3. Results

The results of an attempt to stimulate the virion-associated $Mg^{2+}$-dependent activity with ribosomes are given in table 1. It can be seen that the presence of ribosomes has stimulated the $Mg^{2+}$-dependent activity of NWS to a degree comparable to the $Mn^{2+}$-dependent activity. Ribosomes alone, without the virions, had no polymerase activity. In this experiment no $Mg^{2+}$-dependent activity was found in the absence of ribosomes, but a slight activity, never exceeding 10% of the $Mn^{2+}$ activity, was observed in some other experiments. Ribosomes washed with 1 M KCl in the presence of 1 mM $MgCl_2$ were still fully active. Essentially the same results were obtained with another strain of influenza virus, $A_2$ Singapore 1/57.

The stoichiometry of the stimulation by ribosomes is shown in fig.1. The stimulation of the $Mg^{2+}$-dependent transcriptase activity was dependent on the quantity of ribosomes added to the system, until a plateau was reached estimated at about 50 to 100

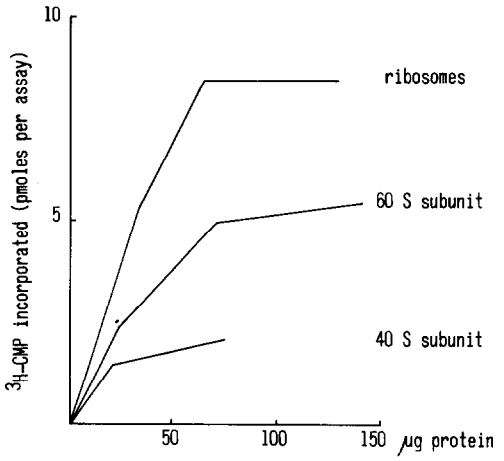


Fig.1. Stimulation of the $Mg^{2+}$-dependent virion-associated transcriptase by ribosomes and ribosomal subunits. Ribosomes were washed with 1 M KCl. Subunit were isolated according to [11] with the puromycin-high salt method. Each assay contained 40 μg protein of NWS virus.

ribosomes per virion. To clarify the stimulatory effect of ribosomes, several factors having some properties in common with the ribosomes have been tested, and they are listed in table 2. These factors have been titrated at several concentrations, as shown in fig.1 for the ribosomal subunits. The factors could be divided into three groups according to their ability to stimulate. The first group includes only ribosomes, which gave full stimulation. The factors of the second group gave between 30 and 60% of the full activation, and include several cellular RNAs or complexes of RNA and protein. The third group, giving no stimulation, includes ribosomal proteins, polyamines, ribohomopolymers, and DNA.

Table 1
Stimulation of the $Mg^{2+}$-dependent virion-associated transcriptase by ribosomes

| Assay conditions | [$^3$H]CMP incorporated (pmoles per mg protein per h) | |
|---|---|---|
| | NWS (50 μg protein) | $A_2$ Singapore 1/57 (72 μg protein) |
| $Mn^{2+}$ 2 mM, KCl 200 mM | 182 | 520 |
| $Mg^{2+}$ 2 mM, KCl 30 mM | 0 | 0 |
| $Mg^{2+}$ 2 mM, KCl 30 mM + ribosomes (109 μg protein) | 196 | 605 |

Table 2
Stimulation of the $Mg^{2+}$-dependent virion-associated transcriptase by various factors

| | |
|---|---|
| Ribosomes (HeLa, calf kidney cultures) | Full stimulation |
| Total cellular RNA[a], rRNA (18 and 28S)[a], ribosomal subunits, *E. coli* ribosomes | Partial stimulation |
| Ribosomal proteins[b], spermine[c], histones[c], poly (A), poly (C), poly (G), poly (I), poly (U)[d], DNA[b] | No stimulation |

Highest concentration assayed: [a]250 μg/ml, [b]350 μg/ml, [c] 50 μg/ml, [d] 75 μg/ml.

Table 2 also shows that the stimulation is rather specific for eukaryotic ribosomes. The stimulated transcriptase activity represents true viral RNA synthesis, since, the four nucleotide bases are incorporated into the product with a base composition corresponding to the plus-stranded viral RNA (unpublished results).

## 4. Discussion

The influenza virion-associated transcriptase is latent in the virion and is activated upon entry of the virion into the cell. The process of activation can be divided into two steps: first, uncoating of the virion, and second, activation of the transcriptase itself. In vitro the second step occurs when $Mn^{2+}$ ions and a high salt concentration are present in the transcriptase assay. These conditions are rather artificial and are not likely to represent the intracellular situation.

The main finding of these experiments is that the latent transcriptase can be activated at low salt concentration and with $Mg^{2+}$ ions, only when ribosomes are present in the system. The ribosomes can be washed with 1 M KCl and they are still active, therefore it is not likely that the ribosomes provide factors participating directly in the enzymatic activity as in the case of the Qβ polymerase. The ribosomes may rather induce a conformational change of the structure of the transcriptase complex which could involve ionic bonds, since it can be induced by increasing the salt concentration in the absence of ribosomes. This activation is probably not unique to the influenza transcriptase, but could be generalized to other viruses such as reovirus, where the activation of the transcriptase also requires an increased $K^+$ concentration [10].

The new conditions for the virion transcriptase assay presented in this paper correspond to the microenvironment that the influenza transcriptase complex finds upon entry into the cytoplasmic matrix: ribosomes, $Mg^{2+}$ and $K^+$ at concentrations compatible with protein synthesis which is important for the coupling of transcription and translation in vitro.

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