# *IN VITRO* SYNTHESIS OF T3 AND T7 RNA POLYMERASE AT LOW MAGNESIUM CONCENTRATION

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## 1. Introduction

In microorganisms a polypeptide chain grows from an initiating N-formyl-methionine residue which is coded for by the starting triplets AUG and GUG. This mechanism seems to be the only specific way of initiation of translation [1]. However, the dependence of initiation on the presence of N-formyl-methionine and the starting triplets is found *in vitro* only at low $Mg^{2+}$ concentrations (4–8 mM); at $Mg^{2+}$ concentrations above 10 mM, protein synthesis is only partially or not at all dependent on fmet-tRNA (2–4).

It would therefore be desirable for the study of *in vitro* DNA-dependent enzyme synthesis to have a cell-free protein synthesizing system which functions at low $Mg^{2+}$ concentration. The cell-free systems published so far, however, all contain $Mg^{2+}$ concentrations above 10 mM, as very little amino acid incorporation takes place at lower Mg concentrations. Polyamines, such as spermidine and putrescine, have been shown to stimulate the activity of RNA polymerase and the binding of aminoacyl-tRNA to ribosomes in low $Mg^{2+}$ concentrations [5, 6]. Therefore, we thought that it should be possible to develop a DNA-dependent enzyme synthesizing system at low $Mg^{2+}$ concentration (5 mM) with the help of spermidine. In this communication we show the effect of spermidine on the *in vitro* DNA-dependent synthesis of T3 and T7 RNA polymerases [7, 8].

## 2. Materials and methods

The protein synthesizing system was isolated from *E. coli* Q13 (RNAase $I^-$, polynucleotide phosphorylase$^-$) [9] according to the method of Traub and Zillig [10] and of Doerfler et al. [11] with some minor modifications: ribosomes were only preincubated and not chromatographed on DEAE-cellulose. Protein fractions and ribosome solutions were stored in liquid nitrogen.

The bacteriophage T3 *am* H95 with an amber mutation in gene 1 (RNA-polymerase gene) was obtained from Dr. R. Hausmann, Spermidine was purchased from Calbiochem. Assay conditions are described in legends of table 1 and table 2.

## 3. Results and discussion

The activity of the protein synthesizing system was studied in two different ways: 1) incorporation of radioactively labeled amino acids ($^{14}$C-leucine) into acid precipitable protein, i.e. total polypeptide synthesis, and 2) synthesis of enzymes (T3 and T7 RNA-polymerases), the enzymatic activities of which were tested in a second assay. In both systems there is an interdependence of $Mg^{2+}$ and spermidine concentration: the higher the $Mg^{2+}$, the lower the spermidine concentrations for optimal activity of the systems, and vice versa.

For the total protein synthesis this interdependence is shown in table 1: the highest activity (here called maximal activity) for T3 DNA-dependent total pro-

Table 1
DNA-dependent *in vitro* protein synthesis at different spermidine and $Mg^{2+}$ concentrations.

| Primer DNA | Spermidine (mM) | $Mg^{2+}$ (mM) | Leucine incorporated at optimal activity (nmoles/0.1 ml) | Maximal activity (%) |
|---|---|---|---|---|
| T3 | – | 14 | 2.4 | 72 |
| | 1 | 10 | 2.9 | 85 |
| | 2 | 8.5 | 3.2 | 95 |
| | 2.5 | 7 | 3.4 | *100* |
| | 3 | 6 | 3.3 | 97 |
| | 4 | 5.5 | 2.5 | 73 |
| | 6 | 4 | 0.8 | 24 |
| T7 | – | 14 | 2.6 | |
| | 3 | 6 | 3.3 | |
| T3 *am* H95 | – | 13.5 | 2.4 | |
| | 3 | 6 | 3.3 | |
| T4 | – | 15 | 2.7 | |
| | 3 | 6.5 | 3.2 | |

The assay mixture of 0.1 ml volume contained: 0.2 mg protein, 1 mg ribosomes, 0.1 mg tRNA, 5 μg DNA, 40 mM Tris-acetate pH 7.9, 1 mM $CaCl_2$, 62 mM $NH_4Cl$, 7 mM KCl, 0.2 mM of 20 amino acids each ($^{14}$C-leucine at 1 mCi/mmoles), 2 mM ATP, CTP, GTP and UTP each, 5 mM phosphoenol pyruvate, 1 μg pyruvate kinase, 1.4 mM dithiothreitol, 0.5 mM 3'5'AMP, 2.7 μg pyridoxine HCl, 2.7 μg nicotine-adenine dinucleotide phosphate, 2.7 μg flavine adenine dinucleotide, 2.7 μg folinic acid, 1.1 μg p-aminobenzoic acid (see [14]) and varied amounts of Mg-acetate and spermidine. Incubation for 15 min at 37°. The values for optimal activity were found by keeping one of the two parameters $-Mg^{2+}$ or spermidine – constant and varying the other. The peak of the resulting curve gives the optimal value of the varied parameter and the optimal activity of the system at the fixed value of the constant parameter. The highest so determined optimal activity was called maximal activity.

Table 2
T3 and T7 DNA-dependent *in vitro* synthesis of RNA-polymerases at different spermidine and $Mg^{2+}$ concentrations.

| Primer DNA | Spermidine (mM) | $Mg^{2+}$ (mM) | UTP incorporated (nmoles) | Maximal activity (%) |
|---|---|---|---|---|
| T3 | – | 11 | 0.8 | 26 |
| | 1 | 8.5 | 1.6 | 53 |
| | 2 | 7 | 2.8 | 90 |
| | 3 | 5 | 3.1 | *100* |
| | 3.5 | 4.5 | 2.6 | 83 |
| | 4 | 4 | 1.3 | 41 |
| | 5 | 2.5 | 0.6 | 19 |
| T7 | – | 11 | 1.1 | 29 |
| | 1 | 9 | 2.6 | 70 |
| | 2 | 6.5 | 3.4 | 92 |
| | 3 | 5 | 3.7 | *100* |
| | 4 | 3.5 | 2.2 | 58 |
| | 5 | 2.5 | 0.4 | 10 |
| T3 *am* H95 | at all concentrations | | < 0.3 | |
| T4 | at all concentrations | | < 0.3 | |

The polymerases were synthesized in the same assay as described in the legend to table 1, except that no radioactively-labeled amino acid was added. In a second assay the activities of the formed polymerases were then tested. This assay contained in 0.2 ml volume: 20 mM Tris-acetate pH 7.9, 23 mM $Mg^{2+}$ acetate, 3 mM spermidine, 0.1 mM EDTA, 2.7 mM dithiothreitol, 10 μg DNA, somewhat less than 1.5 mM ATP, CTP, GTP and UTP each ($^{14}$C-UTP at 0.7 mCi/mmole), 20 μg streptolydigin, 5 μg rifampicin and half the concentration of the other substances of the protein synthesizing assay. Incubation was for 15 min at 37°. The values for optimal and maximal activity (fourth column) were estimated in the same way as described in table 1.

tein synthesis was at 2.5 mM spermidine and 7 mM $Mg^{2+}$. At low spermidine concentration the optimal activity decreased only slowly and was still 72% of the maximal activity in the absence of spermidine. However, at high spermidine concentrations the decrease of activity was more rapid. Above 6 mM spermidine the system was nearly inactive. All 4 DNA's tested (T3, T7, T3 *am* H95 and T4) showed the same activity. Similar results with respect to the interdependence of polyamine and $Mg^{2+}$ concentrations are reported by Takeda [12] in an RNA-dependent cell-free protein synthesizing system, although he did not discuss the relative stimulation of protein synthesis by the polyamines.

The examination of the *in vitro* synthesis of enzymes was more complicated. Since *E. coli* RNA-polymerase is very active in our system and since rifampicin inhibits only free *E. coli* enzyme and streptolydigin is not a very potent inhibitor [13], one has to make sure that the observed effects are not due to artifacts, i.e. residual activity of *E. coli* polymerase. Therefore, we used as control two DNA's which should not promote the synthesis of rifampicin- and streptolydigin-resistent RNA-polymerases; these

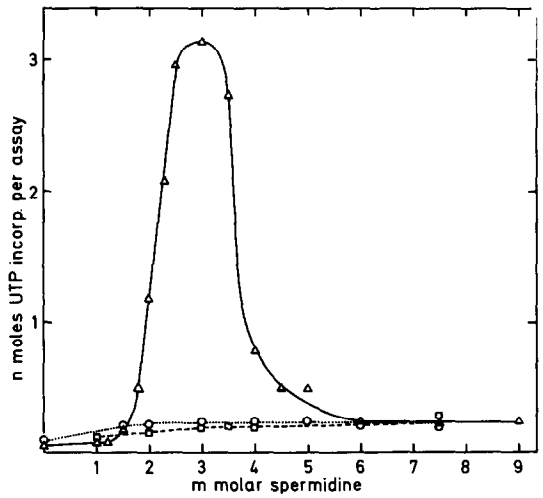


Fig. 1. Synthesis of T3 RNA-polymerases (△—△). Assay as described in legend to table 2 with 5 mM $Mg^{2+}$ as constant parameter. T4-DNA (○· · ·○) and T3 *am* H95-DNA (□ - - -□) as control. Optimal activity here equals maximal activity at 3 mM spermidine and 3.1 nmoles UTP incorporated.

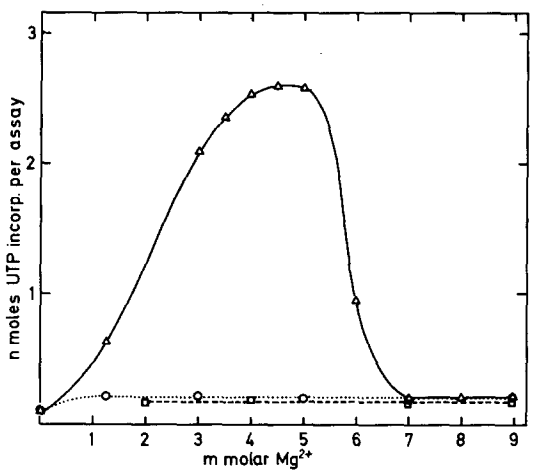


Fig. 2. Synthesis of T3 RNA-polymerase (△—△). Assay as described in legend to table 2 with 3.5 mM spermidine as constant parameter. T4-DNA (○· · ·○) and T3 *am* H95-DNA (□ - - - □) as control. Optimal activity is at 4.5 mM $Mg^{2+}$ and 2.6 nmoles UTP incorporated.

were the DNA's of the bacteriophages T4 and T3 *am* H95 (RNA-polymerase$^-$).

As can be seen from the results of T3 and T7 RNA-polymerase syntheses, summarized in table 2, the requirements for spermidine are much more stringent than in the case where total protein synthesis is measured. In the absence of spermidine the system was working only at $Mg^{2+}$ concentrations above 10 mM and its activity was at best 30% of the maximal activity. It is very difficult to get reproducible results under these conditions: in many experiments we did not find any activity of synthesized enzymes at all. Similar difficulties are reported by other authors [14, 15].

With the addition of 3 mM spermidine the highest yield of active enzyme was obtained at 5 mM $Mg^{2+}$. Under these conditions specific initiation of translation is also optimal. Moreover, the system is now easily reproducible. The difference of the spermidine effect in the total protein and in the enzyme synthesizing system may be due to the fact that the lack of specific initiation at high $Mg^{2+}$ concentrations does not affect the former system so much as the latter one, since each growing polypeptide chain will eventually become acid insoluble, whereas a non-specifically started polypeptide chain will never gain enzymatic activity.

The DNA's of T3 *am* H95 and T4 never exhibited more than 5–6% of the maximal activity of T3 or T7 DNA. These represent the background values which are mainly due to residual activity of *E. coli* RNA-polymerase in the presence of streptolydigin and rifampicin. Addition of chloramphenicol or puromycin or omission of template DNA or ribosomes reduced the background to 2–3% of maximal activity. The activity peak at constant $Mg^{2+}$ concentration and varied spermidine concentrations (fig. 1) covered always a narrower range than the reverse situation (fig. 2). This effect may be due to the increased ambiguity of codons at high concentrations of spermidine, thus leading to translational errors [16].

T7 DNA always promoted the synthesis of more polymerase activity than did T3 DNA, although both DNA's showed the same activity with respect to total protein synthesis (table 1). Besides spermidine, other polyamines like putrescine or spermine showed stimulating ability. Optimal activity was observed in the presence of 17 mM putrescine or 1.5 mM spermine at 5 mM $Mg^{2+}$.

If one assumes a specific activity for pure T7 or T3

RNA-polymerase of 20 μmoles UTP incorporated in 10 min at 37° per mg protein [7, 8] one can calculate that there are about 0.2 μg polymerase synthesized in our 0.1 ml standard assay under optimal conditions when the total protein synthesis is 4 μg per assay; i.e. about 5% of the synthesized proteins is phage-specific RNA-polymerase. These values are about 10–50 times higher than similar data already published [17, 18]. The use of spermidine should offer a considerable improvement of DNA-dependent synthesis of biologically activity proteins.

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