# IN VITRO SYNTHESIS OF DEOXYNUCLEOTIDE KINASE, DIHYDROFOLATE REDUCTASE AND DEOXYCYTIDYLATE HYDROXYMETHYLASE FROM RNA TRANSCRIPTS OF T2 PHAGE DNA[1]

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SUMMARY

RNA transcripts of specified lengths were isolated from an in vitro system containing T2 phage DNA and E. coli RNA polymerase. The informational content of these RNA segments was monitored for deoxynucleotide kinase, dihydrofolate reductase and deoxycytidylate hydroxymethylase with a cell-free protein synthesizing system from E. coli. All three enzymes were translated from RNA chains of 2,300 nucleotides, but deoxycytidylate hydroxymethylase appeared not to be translated from chains of 1,500 nucleotides. Only deoxynucleotide kinase synthesis was directed by transcripts 700-900 nucleotides long. Based on the recently reported subunit molecular weight of this enzyme (1), the gene for deoxynucleotide kinase would appear to be located adjacent to its promoter.

## INTRODUCTION

A recent investigation on the order of expression of five T2 phage-directed enzymes revealed that $H_2$folate reductase, dCMP deaminase, and dTMP synthetase are expressed in the same temporal sequence in vivo as in vitro (2). Such was not the case, however, for deoxynucleotide (HMdCMP)[2] kinase (gene 1) and dCMP hydroxymethylase (gene 42). Thus, while HMdCMP kinase appeared last in vivo, it was synthesized first in vitro, followed by $H_2$folate reductase. This finding is in apparent agreement with studies using sized fragments of DNA as templates in an in vitro protein synthesizing system. Of the five enzymes thus studied, the genes for $H_2$folate reductase and HMdCMP kinase appeared to be closer to their promoters than the genes for the other enzymes (2). From the estimated molecular weights

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[2]Abbreviations used are: HMdCMP, 5-hydroxymethyl deoxycytidylate; SDS, sodium dodecyl sulfate.

of the DNA segments containing the reductase and kinase genes, it was proposed that the minimal average transcriptive unit for these genes should be about 1,500 nucleotides.

To obtain a more accurate assessment of the location of the genes for HMdCMP kinase and $H_2$folate reductase relative to their respective promoters, RNAs of specific sizes were synthesized in an in vitro system containing T2 phage DNA and RNA polymerase. The various RNAs were isolated and translated in a cell-free protein synthesizing system to determine their informational content. This study also included dCMP hydroxymethylase, since like $H_2$folate reductase it appears to qualify as an immediate-early enzyme (2-4).

From the obtained findings, it will be shown that the minimal RNA sizes required for functional HMdCMP kinase, $H_2$folate reductase and dCMP hydroxymethylase synthesis are 800, 1,600 and 2,300 nucleotides, respectively.

## MATERIALS AND METHODS

RNA polymerase and ρ factor: Frozen E. coli B (ATCC 11303) grown to early log phase in high peptone medium (General Biochemicals, Inc.) was used as a source for RNA polymerase and ρ factor. The polymerase was purified by the method of Burgess (5) to a specific activity of 6,900 T2 units/mg (6) and appeared to be at least 90% pure by SDS-acrylamide gel electrophoresis. The procedure of Roberts (7) was used to prepare ρ and was close to homogeneity as judged by SDS-acrylamide gel electrophoresis. RNA synthesis, carried out for 20 min at 37°, was depressed by 60% when ρ was included in the polymerase assay at a concentration of 9 µg/ml (8).

In vitro RNA synthesis: RNA synthesis was carried out at 18° to limit the rate of chain growth. The complete synthesis mixture (5 ml) contained 50 mM Tris-HCl (pH 7.9); 50 mM KCl; 10 mM $MgCl_2$; 0.1 mM dithiothreitol; 0.4 mM each of ATP, GTP, CTP, and UTP; 300 µg of T2 DNA; and 100 µg of RNA polymerase. Rho factor, when used, was added at a final concentration of 9 µg/ml. Following an incubation for 10 min at 18° in the presence of ATP and GTP, synthesis was initiated by the simultaneous addition of UTP, CTP, and rifampicin to a final concentration of 4 µg/ml. The rifampicin was added to limit further RNA chain initiation by the polymerase (9). To determine the size and amount of RNA product, a 0.15-ml aliquot was transferred immediately on initiation of synthesis to a tube at 18° containing 10 µl of 0.35 to 1.0 µCi of [8-$^{14}$C]ATP (44.5 µCi/µmole) (Schwarz/Mann Bioresearch). The shorter the duration of synthesis, the higher the specific activity of ATP used.

Isolation and sizing of in vitro RNA: At indicated times, the large-scale RNA synthesis was terminated by addition of SDS and sodium acetate (pH 5.2) to 1% and 0.1 M, respectively. The RNA was isolated, after addition of 1.8 mg of E. coli ribosomal RNA as carrier, by a modified (10) hot phenol

extraction and ethanolic precipitation procedure (11). The RNA was centrifuged, washed three times with cold 70% ethanol, dried in vacuo at 37°, and dissolved in 0.3 ml of distilled water. After dialysis against distilled water, the concentration of RNA was adjusted to 5 mg/ml ($A_{260}$ = 25/mg/ml). This solution was used as template for in vitro protein synthesis.

Each [$^{14}$C]ATP synthesis was terminated at the same time as the respective large-scale reaction by adding 30 µl of 2.4% SDS and incubating at 37° for 3 min. Ribosomal RNA (200 µg) was added to 0.12 ml of this mixture, which was denatured with formaldehyde in a total volume of 0.5 ml (12). A portion of each treated [$^{14}$C]RNA sample, containing 1,200-2,900 cpm in 0.1 ml, was sedimented through a formaldehyde-containing sucrose density gradient (12) at 4° for 4 hr at 60,000 rpm in a Beckman SW 65LTi rotor. Fifteen-drop fractions were collected in 0.5 ml of water, and the optical markers were located at 260 nm. Ten µg of E. coli tRNA was added to each fraction, followed by 0.5 ml of cold 12% $CCl_3COOH$. Precipitates were collected on Whatman GF/C filters (6) and counted in 10 ml of a toluene-based fluor at 82% efficiency. The recovery of radioactivity from the gradients was 88 to 93%. Molecular weights of RNA were calculated from the measured S values (13).

The total synthesis of oligonucleotide in the original 5-ml incubation mixture was estimated from the acid-stable [$^{14}$C]AMP incorporated in the daughter reaction and the mole fraction (0.3) of AMP in T-even phage mRNA (14). The obtained value was corrected for an 8-10% loss during the isolation procedure. This loss is estimated from the recovery of carrier ribosomal RNA.

In vitro protein synthesis: The conditions used for RNA-directed cell-free enzyme synthesis have been described in detail elsewhere (2). Each reaction mixture (0.2 ml) received 200 to 300 µg of total RNA, of which 10 pmoles of in vitro synthesized RNA product served as the template (calculated from µg RNA/average RNA molecular weight).

Additional methods: HMdCMP kinase, $H_2$folate reductase, and dCMP hydroxymethylase were assayed as described earlier (2). Ribosomal RNA was purified from E. coli B ribosomes by the method of Bolton (15).

## RESULTS

The rate of in vitro RNA synthesis, measured by [$^{14}$C]ATP incorporation, was linear for 20 min and then declined steadily (data not shown). The sedimentation characteristics of formaldehyde-denatured [$^{14}$C]RNA synthesized for various times after initiation are shown in Fig. 1. It is evident from the sharp, symmetrical peaks that the RNA in each case is rather uniform in size. The molecular weight (determined from the relationship $S = 0.05\ M^{0.4}$ [13]) increased linearly with time from $2.4 \times 10^5$ to $8.0 \times 10^5$ at a rate of 2.44 ± 0.06 nucleotides/sec, a value in agreement with that obtained at 37° under similar conditions (12, 16).

For the translation studies, RNAs corresponding to the sizes shown in Fig. 1 were isolated from the large-scale RNA polymerase reaction. In each

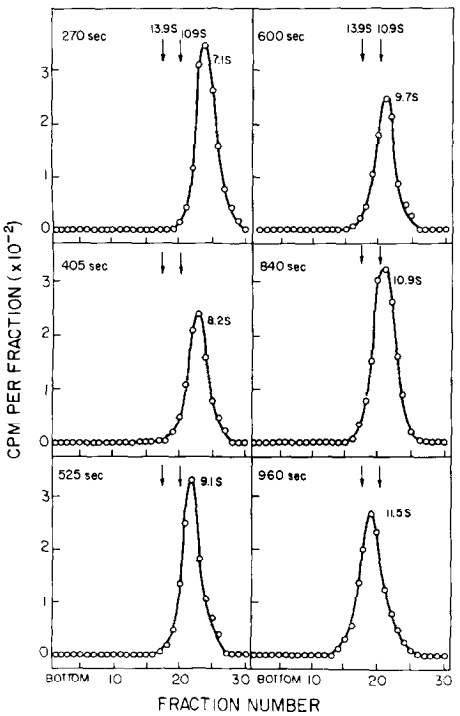


Figure 1. Sedimentation profiles of RNA products synthesized in vitro for varying periods of time using T2 phage DNA as a template. For details see Methods. The arrows indicate the locations of added ribosomal RNA markers.

case, regardless of the lengths of the purified RNA chains, about 10 pmoles saturated the cell-free protein synthesizing system using [$^3$H]leucine incorporation as a measure of protein synthesis. Under these conditions, the total leucine incorporated was a linear function of RNA chain length, with 20 pmoles of leucine incorporated/mg S-30 protein/100 nucleotides. To determine the nature of the information in these RNAs, the proteins that were produced in a cell-free system in response to each RNA were assayed for HMdCMP kinase, $H_2$folate reductase and dCMP hydroxymethylase. As shown in Fig. 2, the level of each enzyme synthesized varied with respect to the others and directly with the length of the RNA template employed.

As a measure of the size dispersion of each sample, the width of the central half of each RNA peak in Fig. 1 was used, and a range about the mean of $\pm$ 250 nucleotides was calculated for RNA chains shorter than

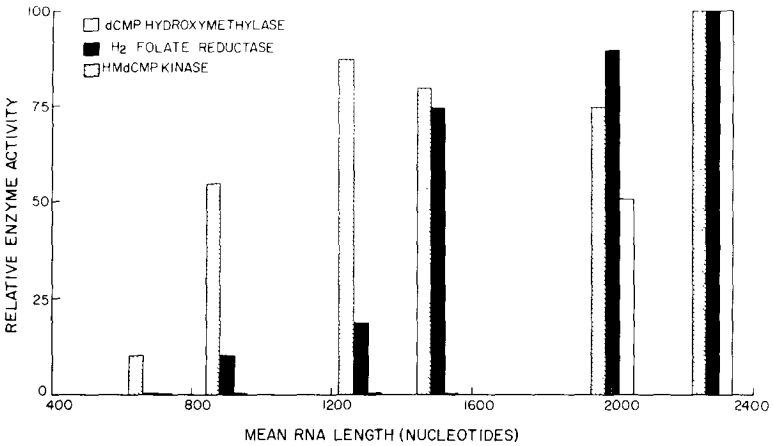


Figure 2. Dependence of enzyme synthesis on the length of RNA transcripts generated in vitro. The protein synthesis is described in Methods. After 20 min incubation at 37°, the reactions were terminated with chloramphenicol and the indicated enzymes were assayed as described previously (2). An activity of 100 corresponds, in milliunits/mg of protein, to 0.27 for HMdCMP kinase, 0.80 for $H_2$folate reductase, and 0.035 for dCMP hydroxymethylase.

1,600 nucleotides. For longer chains a range of ± 400 nucleotides was determined. In all cases, 85-90% of the material in each peak was shorter than the mean size of the next larger sample. From these data, it was estimated that the minimum functional transcript size for HMdCMP kinase is about 800 nucleotides; for $H_2$folate reductase, 1,600 nucleotides; and for dCMP hydroxymethylase, 2,300 nucleotides. Intermediate RNA sizes of 650, 1,150, 1,700 and 2,150 nucleotides yielded similar results (data not shown).

When the in vitro RNA synthesis was extended beyond 30 min, polydispersity appeared in the sedimentation profiles, as evidenced by conspicuous shoulders at 15S and 18S and a broad peak at 12-13S. In the presence of the termination protein ρ, however, the RNA chain length was limited to 8.5S and 10.5S species, corresponding to mean lengths of 1,100 and 1,850 nucleotides, respectively (Table I). Comparison of the template capacities of RNAs synthesized for 30 min in the presence and absence of ρ indicates that ρ does not prevent the transcription of mRNA for any of the enzymes studied. As might be expected from the size distribution of the ρ RNA product, the template

Table I. Template capacity of RNA synthesized in vitro in the presence and absence of ρ factor

| RNA synthesis conditions[a] | RNA size (S value) | In vitro translation system[b] | | | |
|---|---|---|---|---|---|
| | | [$^3$H]leucine incorporation in 20 min (nmoles/mg) | HMdCMP kinase (milliunits/mg) | $H_2$folate reductase (milliunits/mg) | dCMP hydroxymethylase (milliunits/mg) |
| +ρ | 8.5; 10.5 | 0.12 | 0.17 | 0.70 | 0.010 |
| -ρ | 10.5-15 | 0.32 | 0.15 | 0.75 | 0.027 |

[a] RNA synthesis was for 30 min at 18° as described in Methods.

[b] The 0.2-ml cell-free protein synthesis system was primed with 5.7 μg of 30-min ρ-stop RNA, equivalent to 10.7 pmoles of oligonucleotide ($N_{average}$ = 1,500), or with 9 μg of 30-min RNA, equivalent to 9 pmoles of oligonucleotide ($N_{average}$ = 2,900).

content for dCMP hydroxymethylase was reduced but not that for $H_2$folate reductase or HMdCMP kinase (Table I).

## DISCUSSION

Current concepts of the initiation of RNA synthesis from rifampicin-resistant RNA polymerase holoenzyme complexes suggest that the synthesis begins at specific promoter sites (17-19). Thus correlating the length of RNA chains synthesized with their information content should give a measure of the relative distance of specific genes from their promoters. Evidence in support of this approach is indicated by the direct relationship between the length of input RNA and the encoded enzyme-specific information (Fig. 2). Although the gene for HMdCMP kinase could, on the basis of promoter distances alone, be located on the same transcript as that for $H_2$folate reductase or dCMP hydroxymethylase, the actual map locations of these genes precludes that possibility (20). It appears therefore, that the information for the three enzymes reported here must be present on transcripts initiated at three separate promoter sites.

The finding that the kinase is translated from an RNA segment of

about 800 nucleotides suggests that this gene may be promoter proximal and confirms the DNA shearing studies reported earlier (2) on the proximity of $H_2$folate reductase and HMdCMP kinase to their respective promoters. The former data are in good agreement with recent studies indicating that T4 phage HMdCMP kinase is composed of two identical 23,000 molecular weight subunits (1), since a protein of this size would only require a transcript of about 700 nucleotides. Any difference in size between the HMdCMP kinase gene and the active transcripts obtained in these studies may be accounted for by the promoter nucleotide sequence (21) or even by experimental error.

Both ρ factor in vitro and chloramphenicol in vivo promote the formation of immediate-early RNAs which appear identical by the criteria of hybridization and size (1,500-2,000 nucleotides) (8, 22). Their information content, however, appears to differ, since the chloramphenicol RNA isolated from cells infected with either T2 or T4 phage (2, 23) fail to direct the cell-free synthesis of HMdCMP kinase, while the RNA synthesized in vitro in the presence of ρ is functional for this enzyme (Table I). The reason for this difference remains to be clarified.

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