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In vitro methylation of nascent reovirus mRNA by a virion-associated methyl transferase

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ABSTRACT

Chymotrypsin-derived cores, but not virions, catalyze the transfer of methyl groups from S-adenosyl methionine (SAM) to nascent mRNA synthesized in vitro by the core polymerase. The reaction requires Mg^{++}, and is dependent on the presence of all 4 ribonucleoside triphosphates (rNTPs). Methylation proceeds optimally at 51°C. All ten species of mRNA become methylated during transcription and it is estimated that one methyl residue is incorporated per RNA chain. Experiments designed to determine the location of the methylated nucleotide clearly demonstrate that methylation occurs exclusively at the 5' ends of nascent mRNA.

INTRODUCTION

The double capsid of reovirions is composed of an outer protein shell and an inner ribonucleoprotein core which contains the genomic segments of double-stranded RNA[1,2]. Treatment of whole virions with chymotrypsin removes the outer capsid leaving intact cores. Of the seven polypeptides found in virions, four are located in the core, while the remaining three make up the outer capsid[3]. Cores have previously been shown to possess two enzymatic activities: a phosphohydrolase[4,5] and an RNA polymerase[6,7]. The polymerase transcribes all ten segments of the viral genome into their corresponding mRNAs in vitro. Each species of mRNA has been shown to contain ppGp at its 5' end and it has been suggested that the terminus is diphoshorylated as a result of the virion associated phosphohydrolase activity[8]. Although neither the polymerase nor the phosphohydrolase is expressed in whole virions, both become active when the outer capsid is removed by chymotrypsin. In contrast, a poly A polymerase which is active in whole virions, is destroyed by

chymotrypsin and is therefore presumed to reside in the outer capsid[9].

The purpose of this paper is to describe an RNA methylase which is found in association with purified preparations of reovirus. A similar activity has recently been reported for cytoplasmic polyhedrosis virus (CPV) another member of the diplorna virus group[10]. The experiments described here were designed to investigate the general properties of the enzyme and to specifically determine 1) whether the methylase is associated with the core or the outer capsid of purified virions 2) the number of methyl groups incorporated into each RNA molecule and 3) the location of the methylated nucleotide in nascent mRNA chains.

MATERIALS AND METHODS

Cells and Virus. Mouse L-cells were grown as described previously[11] and infected with the Dearing strain of reovirus type 3 as described by Smith et al[3].

Digestion of Virus with Chymotrypsin. Virus, at a concentration of 3mg/ml, was incubated with 100 µg/ml chymotrypsin for forty-five minutes at 37°C in 0.5 M Tris-Cl buffer, pH 8.3. Cores produced in this way were used immediately in enzyme assays.

Assay of Methyl Transferase Activity. The reaction mixture contained in a total volume of 0.75 ml; 2 µmoles each of ATP, CTP, GTP, UTP, 9 µmoles $MgCl_2$, 50 µmoles phosphoenol pyruvate, 100 µg pyruvate kinase, 75 µmoles Tris-Cl pH 8.3, 1.8 µg actinomycin D, 3 nmoles S-adenosyl-L-methionine [Methyl-^{3}H] (specific activity - 8.5 Ci/mmole), and 440 µg of chymotrypsin-treated virus. If RNA synthesis was to be measured as well, the amount of one of the nucleoside triphosphates was decreased nine-fold and adjusted to a final specific activity of 3-4mCi/mmole using the appropriate radioactive precursor. In cases where smaller reactions mixtures were used i.e., less than 0.75 ml, all substrates were reduced in the same proportion as the volume of the reaction mixture. Incubation was at 44°C and reactions were

terminated using 10% TCA. Samples were collected on membrane filters (Millipore Corp.), dried and the radioactivity determined in an SL-30 Intertechnique liquid scintillation counter (Canatech Inc.) equipped with a Multi-20 computer.

Synthesis and Purification of Methylated mRNA. The conditions used for the synthesis of methylated mRNA were identical to those described above except that 500 µg of bentonite was included in the reaction mixture. After four hours at 44°, the mixture was centrifuged for forty-five minutes at 30,000 g in a Sorvall refrigerated centrifuge. The supernatant was filtered through a Sephadex G-100 column in 20 mM sodium acetate buffer pH 5.2, 1 mM ethylenediamine tetraacetate (EDTA) and 0.1% sodium dodecyl sulphate (SDS). The void volume was collected and dialyzed against 50 mM Tris-Cl buffer pH 8.0, containing 10 mM NaCl, 1 mM EDTA, and 0.1% SDS.

Size of the Methylated RNA. Samples were prepared for sucrose gradient sedimentation by heating at 37°C for thirty minutes in the presence of 50 mM sodium acetate pH 5.2, 10 mM EDTA, 0.1% SDS and 0.5 M urea. The RNA was layered over a 10-42% sucrose gradient containing 50 mM sodium acetate pH 5.2, 1 mM EDTA, 0.5% SDS, and centrifuged at 25°C in an SW 40 rotor for sixteen hours at 36,000 rpm. Gradient fractions were collected from the bottom of the centrifuge tube and the radioactivity in each fraction was determined using Aquasol (New England Nuclear).

Polyacrylamide Gel Electrophoresis. mRNA was analyzed in 2% acrylamide 0.6% agarose-6 M urea gels as described by Floyd et al[12].

Alkali Hydrolysis and DEAE Cellulose Chromatography. RNA was hydrolyzed in 0.3 N KOH at 37°C for 18 hrs. Samples were neutralized with DOWEX-50 (H^+) and analyzed on DEAE-cellulose columns in 7 M urea, as described previously[8]. A pancreatic ribonuclease digest of wheat germ ribosomal RNA was used as chain length marker.

RESULTS

Detection of Methyl Transferase Activity. Virions were digested with chymotrypsin and assayed for methyl transferase activity as described in Materials and Methods. For comparison, RNA polymerase was measured simultaneously by following the incorporation of ^{14}C-UMP into RNA.

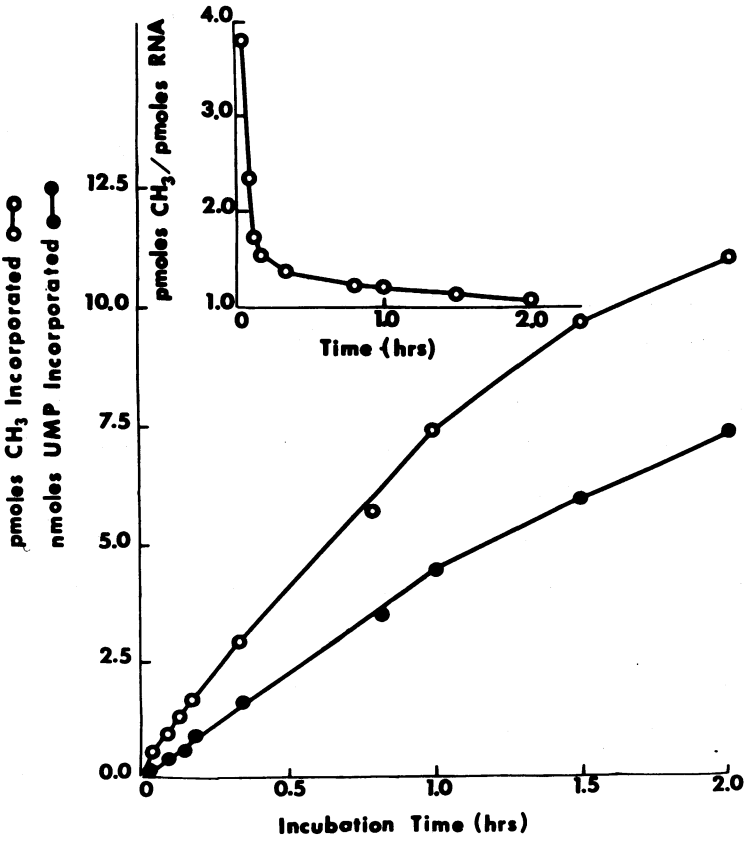

Figure 1. Incorporation of ^{3}H-methyl and ^{14}C-UMP into TCA insoluble material. Values for pmoles RNA (inset) were determined for RNA containing 2500 nucleotides.

The results (Figure 1 and Table I) show that a methyl transferase is latent within the virion and becomes activated by chymotrypsin treatment. In the presence of all four rNTP's and an ATP generating system, the transfer of

methyl residues from SAM into TCA insoluble material proceeds for several hours. As shown in Table I there is little or no activity when only ATP is added to the reaction mixture or when whole virions are used as the source of enzyme. From these results it is concluded that, like the polymerase and phosphohydrolase, the methylase of the virion resides in a chymotrypsin resistant core.

TABLE I

Transcription-dependent methylase activity of chymotrypsin-derived cores

Sample	TCA insoluble CPM[1]
Virions + chymotrypsin + 4 rNTP's	1500
Virions + 4 rNTP's	100
Virions + chymotrypsin + ATP	30

[1] Represents incorporation of ^{3}H-methyl during thirty minutes of incubation. The volume of the reaction mixture in each case was 0.075 ml. (TCA = trichloroacetic acid).

Further investigation of the properties of the enzyme revealed that divalent cations are absolutely required for activity, with magnesium being preferred over manganese. Alternatively, monovalent cations such as K^{+} and Na^{+} were found to be generally inhibitory. The pH and temperature optima lie between 7.5 - 8.0 and 51° to 56°C respectively (Figure 2a-d) and as expected, the extent of methylation is directly proportional to the amount of virus used (Figure 3).

The optimum SAM concentration was determined in the experiment illustrated in Figure 4. Amounts of SAM chosen from the flat portion of the curve were used in all subsequent experiments.

Characterization of the Methylated Product. The dependence of the methylation reaction on the presence of all four rNTP's strongly suggested that methylation was occurring on nascent mRNA chains. In order to test this

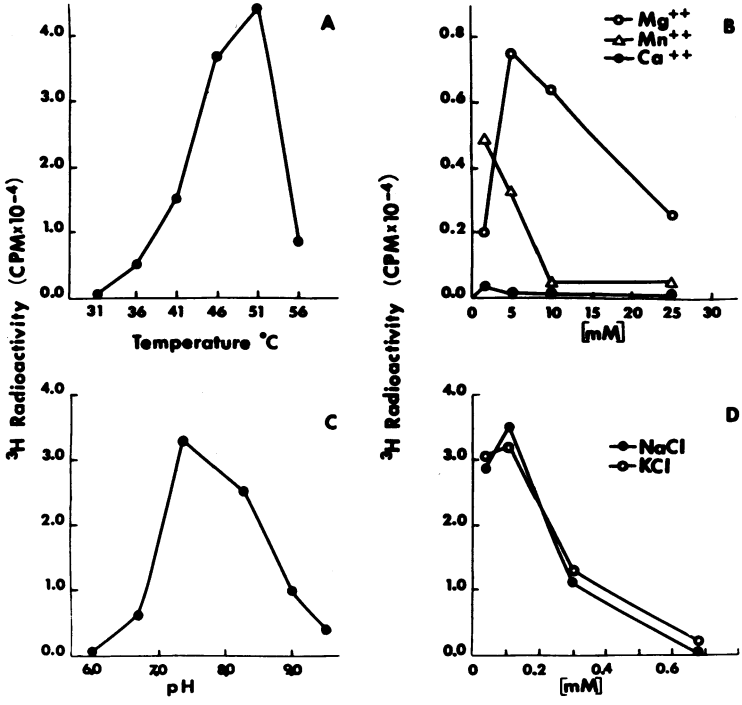

Figure 2. Dependence of methylation on a) temperature b) divalent cations c) pH and d) monovalent cations. All experimental points represent ^{3}H-methyl incorporation during thirty minutes of incubation. Except for the parameters which were varied experimentally, all the assay conditions were those described in Materials and Methods. Reaction mixtures were 0.125 ml each except for those in figure b) where they were 0.075 ml each.

hypothesis, RNA was synthesized in the presence of ^{3}H-SAM and α-^{32}P-UTP (New England Nuclear) and purified as described in Materials and Methods, and an aliquot was tested for its sensitivity to digestion with 5 µg/ml of pancreatic ribonuclease in the presence of 0.3 M NaCl. As expected, ribonuclease treatment rendered all of the radioactivity soluble in 5% TCA. In addition, the methylated products sediment together with the three size classes of *in vitro* synthesized mRNA in sucrose gradients

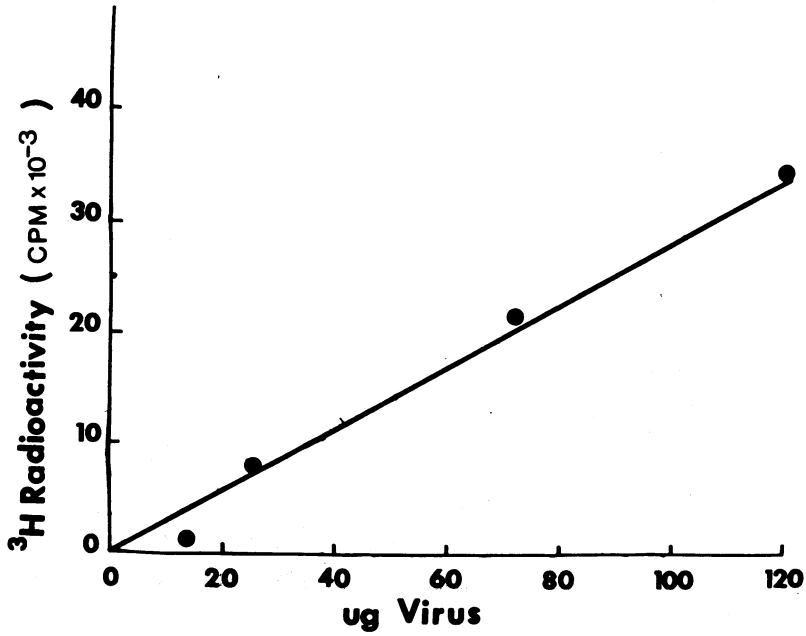

Figure 3. Dependence of methylation on the amount of virus used. 0.125 ml reaction mixtures were incubated for sixty minutes and the TCA insoluble CPM determined.

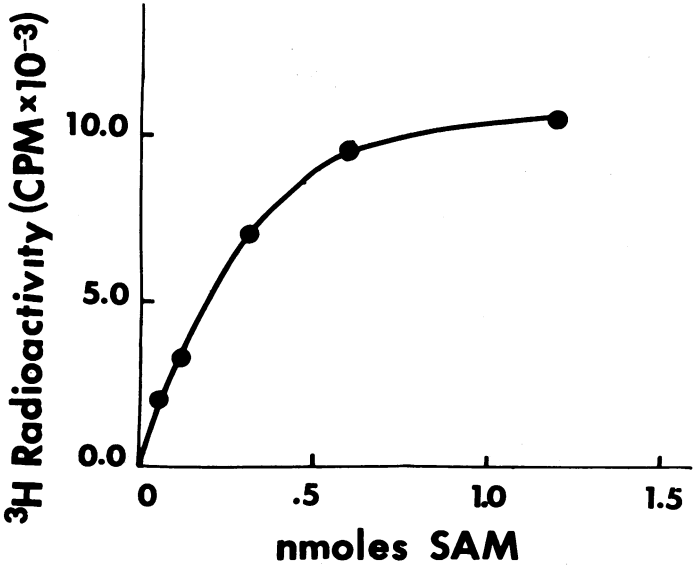

Figure 4. Effect of SAM concentration on the extent of methylation. 150 µg chymotrypsin treated virus was incubated at 44° for 2 hr with varying amounts of SAM (final spec. activity = 0.81 Ci/mmole). The volume of each reaction mixture was 0.30 ml.

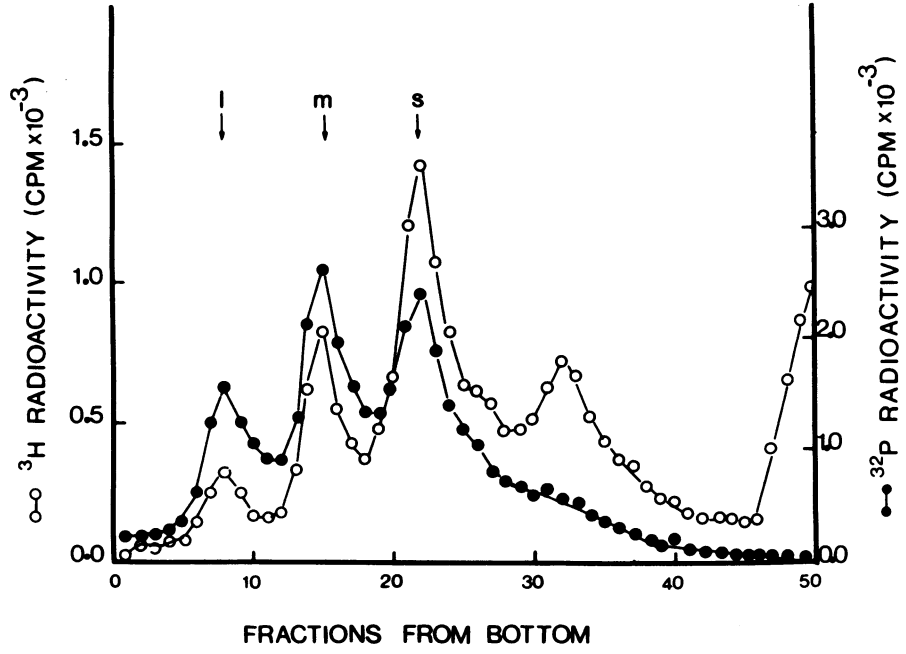

Figure 5. Sedimentation of ^{3}H-methyl RNA labelled internally with α-^{32}P-UTP. l, m, and s denote the 24S, 18S and 14S species of mRNA respectively.

(Figure 5). We concluded from these results that methylation occurs on nascent mRNA which is synthesized *in vitro* by the core polymerase.

We next determined the number of methyl groups being introduced into each RNA molecule. From the data presented in Figure 1 we calculated that one methyl group is transferred for every 2500 nucleotides of newly synthesized RNA. Since the ten segments of mRNA are made up of chains varying in length from 1000 to 3600 nucleotides[8], an average chain length would be composed of approximately 2200 to 2500 nucleotides. Thus, on the average, one methyl group is introduced into each RNA molecule at some point during its synthesis. This estimate was confirmed by analyzing ^{3}H-methyl RNA in polyacrylamide gels. As illustrated in Figure 6, each of the ten species of mRNA has a methyl group associated with it.

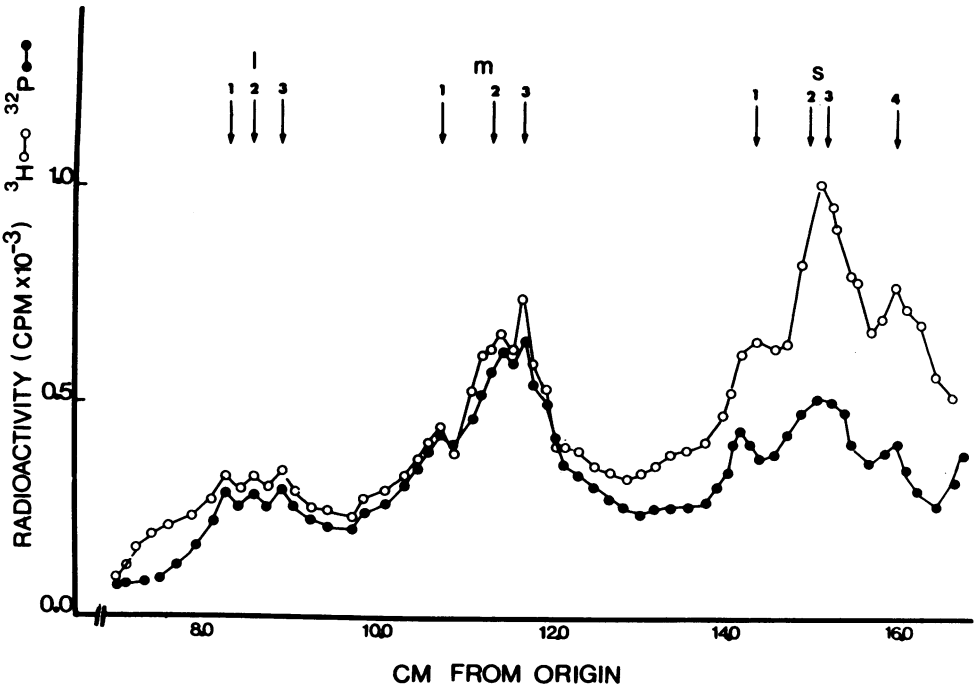

Figure 6. Polyacrylamide gel electrophoresis of mRNA labeled with α-^{32}P-UTP and ^{3}H-methyl. Electrophoresis is from left to right.

Determination of the Site of Methylation. During the course of these studies we observed that SAM enhances the rate of transcription almost three-fold (data not shown). We reasoned that this result might be explained if a methylation event which facilitates initiation occurs at the 5' end of nascent mRNA. In agreement with this hypothesis it was observed that during the *in vitro* synthesis of methylated mRNA, the ratio of the number of methyl groups incorporated into RNA, to the total amount of RNA synthesized, decreases as a function of the time of incubation (Figure 1, inset). This result suggested that methylation occurs early in the synthesis of nascent RNA chains and would mean that the site of methylation is at or near the 5' end. In order to test this hypothesis directly, methylated RNA was hydrolyzed with alkali and the hydrolysate analyzed on a DEAE-cellulose column as described in Materials and Methods. The elution profile is illustrated

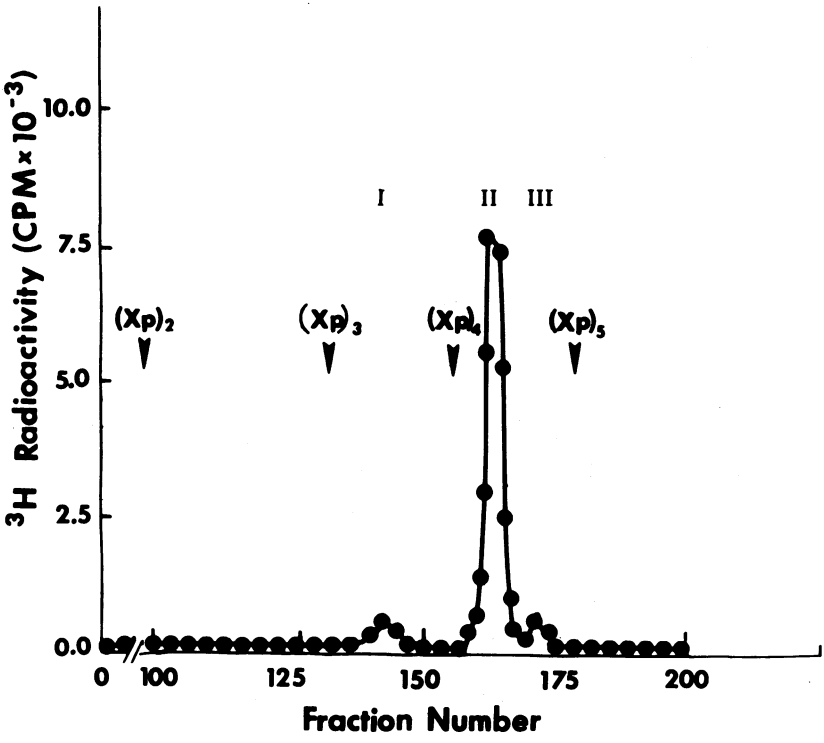

Figure 7. DEAE-cellulose chromatography of methylated nucleotides. $(Xp)_n$ denotes the position of the chain length markers obtained from a ribonuclease A digest of ribosomal RNA.

in Figure 7. Greater than ninety percent of the radioactivity eluted at the position expected for material having a net charge of -6. A single methylation event on the 2'-OH position of internal ribose moieties would yield alkali-resistant dinucleotides. However, no ^{3}H radioactivity eluted in this region so we tentatively conclude that the methylated nucleotide is polyphosphorylated and originates exclusively from the 5' end.

The methylation reaction was further characterized as described below and in the legend to Figure 8. Unmethylated mRNA was synthesized and purified and then tested for its ability to act as substrate for the methylating enzyme of the core. For comparison the methylation of nascent mRNA by an equivalent amount of enzyme was determined in a parallel experiment.

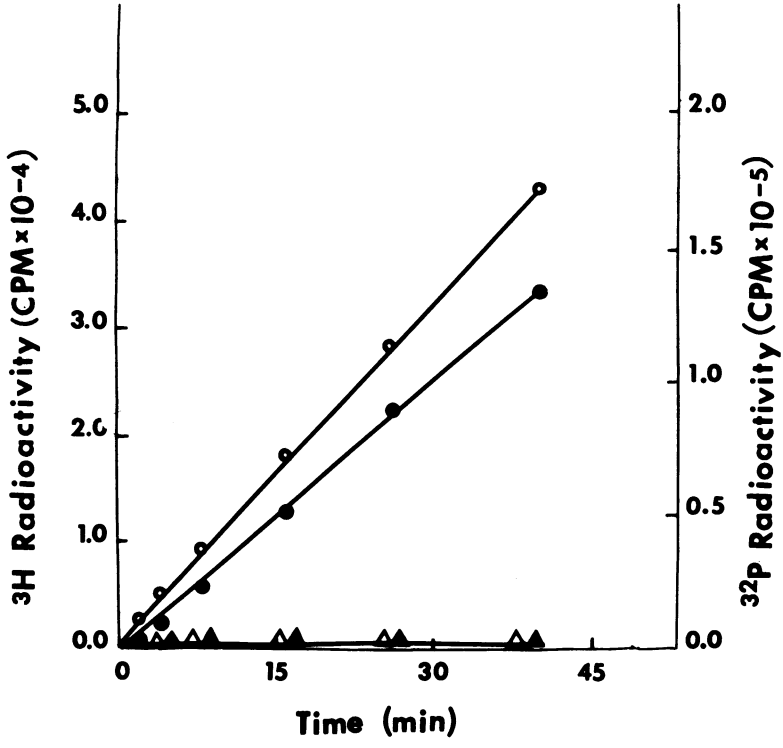

Figure 8. Inability of chymotrypsin cores to methylate exogenous mRNA. Two separate methylase reaction mixtures, 0.75 ml each, containing either 50 µgm unmethylated RNA, together with ATP and α-^{32}P-UTP (incomplete system) or 0 µgm unmethylated RNA with ATP, CTP, GTP,and α-^{32}P-UTP (complete system) were incubated at 44°C and the acid insoluble radioactivity determined. The assay conditions were those described in Materials and Methods except as outlined above. The specific activity of α-^{32}P-UTP was 15 mCi/mmole. Open and closed circles; incorporation of ^{3}H-methyl and α-^{32}P-UMP respectively into nascent mRNA (complete system). Open and closed triangles; incorporation of ^{3}H-methyl and α-^{32}P-UMP respectively in the presence of exogenous mRNA (incomplete system).

As shown in Figure 8, mRNA supplied exogenously remained unmethylated while in a parallel reaction almost 7 picomoles of ^{3}H-methyl became associated with newly synthesized RNA after forty minutes of incubation. Assuming an average molecular weight of 8 x 10^{5} daltons for the RNA, it can be calculated from the incorporation of α-^{32}P-UMP that approximately 6.5 picomoles of nascent mRNA was produced, thus confirming the previous

estimate of one methyl group per RNA chain. These results indicate that once extruded from the core, mRNA is no longer suitable as a substrate for the core methylase. It is interesting in this respect that the phosphohydrolase, another core-associated enzyme, also acts only on nascent polynucleotide[8].

DISCUSSION

The experiments described in this paper show that chymotrypsin-derived cores catalyze the methylation of nascent mRNA *in vitro*. In addition, we have shown that methylation occurs at the 5' terminus and that each of the ten species of mRNA is methylated during *in vitro* transcription. Although methylation enhances the rate of transcription 2 to 3 fold, the stimulation was by no means as marked as that reported recently for CPV[10]. Methylation of reovirus mRNA is highly specific, affecting on the average only one nucleotide in 2500. A similar degree of specificity is found in L-cell mRNA where only 0.22 percent of the nucleotide residues are methylated[13].

Properties of the methylase, such as divalent cation requirement, pH and the unusual temperature optima are remarkably similar to the viral transcriptase enzyme. This is not surprising in view of the dependence of methylation on transcription (Figure 1 and Table I). However, since methyl-deficient reovirus RNA synthesized *in vitro* was not a substrate for the methylase we were unable to study methylation independently of simultaneous transcription.

Evidence presented in Figure 1 (insert) suggests that methylation occurs very early in the synthesis of nascent RNA and that on the average, each mRNA molecule receives only one methyl residue. This is supported further by the fact that all mRNA species are methylated essentially to the same extent (Figure 6). Taken together, these data suggest that methylation may occur concomitantly with the initiation of mRNA synthesis.

Consistent with this notion is the observation that, following alkali hydrolysis, the methylated derivative is recovered as a polyphosphrylated nucleotide which is eluted from a DEAE cellulose column between the tetra-nucleotide (-5 charge) and the pentanucleotide (-6 charge) markers (Fig. 7).

In a preliminary attempt to determine its structure, an aliquot of the methylated derivative was treated with alkaline phosphatase and rechromatographed on DEAE cellulose. All of the ^{3}H-methyl radioactivity was recovered in a single peak which eluted between the dinucleotide (-3 charge) and trinucleotide (-4 charge) markers (K. Hastings, S. Millward unpublished observations), suggesting that a majority of the phosphate groups reside in phosphatase resistant phosphodiester linkages. Experiments to determine the exact structure of the methylated species are presently in progress.

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Note Added in Proof.

Subsequent to submitting this report two papers appeared describing a methyl transferase activity in reovirus (A.J. Shatkin 1974 Proc. Natl. Acad. Sci. 71 3204-3207) and in vaccinia virus (C.M. Wei and B. Moss 1974, Proc. Natl. Acad. Sci. 71, 3014-3018).

*M. Faust is the holder of a Medical Research Council Studentship (Ottawa).
/Medical Research Council Scholar (Ottawa).

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