Ribonucleic Acid Polymerase Reactions with Methylated Polycytidylic Acid Templates*

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SUMMARY

Partially methylated polycytidylic acids were used as templates in an RNA-dependent RNA polymerase reaction with enzyme isolated from Micrococcus lysodeikticus. The presence of methylated cytidylic acid residues in the template led to the formation of a copolymer of uridylic and guanylic acids. The composition of this product copolymer was dependent on the extent of methylation of the polycytidylic acid template.

The mutagenic and carcinogenic effects of alkylating agents have been reviewed recently by several authors (1-3). Since most alkylating agents attack guanine preferentially in both DNA and RNA, molecular explanations for such effects have often centered on alterations in the structure of this particular base. However, other nucleic acid bases are often attacked as well. Such alterations in the structure of RNA could lead to mispairing during the replication of RNA or during the process of translation, with the consequent transmission of incorrect genetic information in either step.

Previously (4, 5), we have investigated possible effects on translation by studying protein synthesis in a cell-free system with methylated polynucleotides as synthetic messengers. These investigations and those of Abell, Rosini, and Rameur (6) have shown that the ability of synthetic polynucleotides to direct protein synthesis was decreased by alkylation, but that no qualitatively different amino acids were incorporated under the influence of the alkylated polynucleotides investigated.

Therefore, we have undertaken a study of the RNA-directed RNA polymerase reaction to determine whether the composition of newly synthesized RNA is affected by the methylation of template polynucleotides. Initial experiments with poly CG templates indicated that the newly synthesized polynucleotides probably had a different composition when the templates were methylated. However, it was not clear whether this reflected changes in the guanine or in the cytosine bases, since both were methylated under the experimental conditions (7). In subsequent studies with poly C and methylated poly C, we have discovered that methylated cytosine promotes the incorporation of UTP into a copolymer of guanylic and uridylic acids in the presence of GTP and UTP. It is this phenomenon that we wish to describe in detail in this publication.

The RNA polymerase system has been investigated extensively by other workers interested in different chemical and physical mutagens. Belman, Huang, Levine, and Troll (8) studied the effects of hydroxylamine on DNA template, and other investigators have studied the effects of ultraviolet irradiation on DNA template (9, 10) and on synthetic polynucleotides (11, 12). Recently, Phillips, Brown, Adman, and Grossman (13) and Wilson and Caicuts (14) have shown that hydroxylamine treatment of poly C leads to the synthesis of poly AG by RNA polymerase, and Phillips, Brown, and Grossman (15) have obtained similar results with methoxyamine-treated poly C. In many respects, the studies reported here are similar to those with hydroxyamine- and methoxyamine-modified poly C, except that methylated poly C leads to the incorporation of UTP instead of ATP into the newly synthesized polymer.

EXPERIMENTAL PROCEDURE

Polynucleotide Templates—All experiments were performed on one sample of poly C obtained from Miles Laboratories, Inc., Elkhart, Indiana. The weight average molecular weight of this polymer was 210,000, as determined by measurements on a Brice-Phoenix light scattering photometer (7). The intensity of scattered light remained stable for several hours at room temperature during these determinations, showing that no detectable nuclease activity was present.

Methylation reactions were carried out at 30° in cacodylate buffer (pH 7, ionic strength 0.2) with methylmethanesulfonate obtained from Eastman Kodak Company, Rochester, New York. This compound was dissolved in buffer and filtered through a
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Gelboran VF filters directly into a light scattering cell containing buffered poly C solution. In these reactions, the final polymer concentration was 1.0 mg per ml and the reaction time was 1½ hours; methylmethanesulfonate concentration was 5.7 mg per ml for the preparation of methyl poly C No. 1, and 12.6 mg per ml for the preparation of methyl poly C No. 2. Continuous monitoring of the intensity of scattered light indicated that no degradation of the partially methylated poly C had occurred. The polymer was precipitated with 3 volumes of 95% ethanol, desolvated in water, dialyzed in the cold against 0.15 M NaCl, 0.015 M sodium citrate, 0.1 M NaF0, and distilled water, and dried by lyophilization.

As reported previously (7), hydrolysis of the methylated polymers with 1 x HCl for 30 min at 100° liberated 3-methylcytidylic acid as the only detectable derivative, while hydrolysis with 70% HClO₄ for 2 hours at 100° liberated 3-methylcytidine.

These compounds had the ultraviolet spectra and paper chromatographic properties reported for these derivatives by Brookes and Lawley (16) and by Lawley and Brookes (17). They were identified further by conversion with alkali to 3-methyluridylic acid and 3-methyluracil, respectively.

Base compositions were determined after hydrolysis with 1 x HCl as described above. Hydrolysates, containing approximately 2 x 10⁶ units, were applied to a DEAE-cellulose column, 1 x 39 cm, and eluted with an exponential 0.01 to 0.1 M gradient of triethylammonium bicarbonate, pH 8.6; 3-methylcytidylic acid was eluted ahead of cytidylic acid. The eluant was pumped at a constant rate (0.5 ml per min) through a 0.2-ml flow cell, while optical density was monitored at 271 mp with a Beckman DB spectrophotometer. The extinction coefficient of 3-methylcytidylic acid at the absorption maximum (266 mp) was obtained for the pK value of 3-methylcytidylic acid, and 9.0 was obtained for the pK value of 3-methylcytidylic acid, and was corrected to pH 8.6 by titration in a Cary model 15 spectrophotometer. From this titration, a value of 9.7 x 10⁻³ for the extinction coefficient at 271 mp and pH 8.6 was obtained for the pK value of 3-methylcytidylic acid, and was corrected to pH 8.6 by titration in a Cary model 15 spectrophotometer. From this titration, a value of 9.7 x 10⁻³ for the extinction coefficient at 271 mp and pH 8.6 was assumed to be the same in alkali as given by Brookes and Lawley (16) for cytidylic acid at the absorption maximum (266 mp).

The polymer was precipitated with 3 volumes of 95% ethanol, desolvated in water, dialyzed in the cold against 0.15 M NaCl, 0.015 M sodium citrate, 0.1 M NaF0, and distilled water, and dried by lyophilization.

Composition of Products—To determine the composition of the products, 1 ml reaction mixtures containing 14C-labeled UTP and GTP at equal specific activities were incubated at 25° for 60 min. The polymer was precipitated with trichloracetic acid-pyrophosphate in the presence of 500 μg of yeast RNA, then suspended and centrifuged successively with five washes of 5% trichloracetic acid, two washes of 95% ethanol, one wash of 1:1 ethanol-ether, and one wash of ether. After the dry precipitate was digested at 37° for 18 hours in 0.1 ml of 0.3 N KOH, 2 μl of 10 N HClO₄ were added, the KClO₄ was removed by centrifugation, and the nucleotide mixture was concentrated and spotted on a Whatman No. 1 filter paper strip. Electrophoresis at 15 volts per cm in 0.025 M glycerine buffer, pH 2.8, separated the nucleotides into three well defined areas: combined AMP-CMP, GMP, and UMP.

The paper was dried, and the various areas were identified with ultraviolet light, cut out, transferred to vials, and counted in a scintillation counter. The average composition of the newly polymerized product was calculated from the relative number of UMP and GMP counts. Since total recovery of radioactivity was variable with the extensive washing procedure, only relative frequencies are strictly comparable among different incorporation experiments.

“Nearest Neighbor” Analysis—Separate reaction mixtures containing α-32P-labeled UTP or GTP were incubated and the product was precipitated with trichloracetic acid; the polymer was then digested and subjected to electrophoresis as described above. Again, the UMP and GMP areas were counted and from these the relative frequencies of uridine-uridine to guanosine-uridine and uridine-guanosine to guanosine-guanosine sequences were calculated.

RESULTS

As might be anticipated, methylation decreased the ability of poly C to act as template for the formation of polyguanylic acid. This effect is shown in Fig. 1, and could indicate that methylated cytosine either acts as template for a base other than guanine or interrupts the polymerization completely. In the absence of other nucleoside triphosphates, similar results would be predicted in either case.

Initial evidence that a copolymer might be formed was obtained by adding unlabeled UTP to the reaction mixture. Although this resulted in a partial recovery of the GTP incorporation when methylated poly C was used as template, such experiments are difficult to interpret since there is a smaller effect, which may be related to the Mn++ to triphosphate ratio, on similar polymerizations in the presence of poly C.

Direct evidence for the polymerization of UTP was obtained by including 14C-UTP in the reaction mixtures. These experiments are shown in Fig. 2, which shows the ability of methylated poly C template to promote UTP incorporation. Control experiments established the dependence of this incorporation on incubation with active enzyme in the presence of Mn++. It was still

1 Chemical Abstracts numbering system is used here; Brookes and Lawley (16) refer to these compounds as 1-methylcytidylic acid and 1-methylcytidine, respectively.

possible, however, that the methylated template in some way stimulated the separate formation of polyuridylic acid instead of poly UG. In fact, the slight background incorporation in the presence of poly C may represent formation of some polyuridylic acid.

Two types of experiments indicated that a true copolymer was probably being formed. First, the incorporation of UTP in the presence of methylated poly C shown in Fig. 2 was dependent on the presence of GTP in the reaction mixture. Second, kinetic analysis showed incorporation of both UTP and GTP throughout the incubation period.

Conclusive evidence for copolymer formation was obtained from the nearest neighbor analysis shown in Table I. The columns labeled guanosine-guanosine and uridine-guanosine measure the relative frequency of these sequences as determined from incorporations with α-32P-GTP; the columns labeled guanosine-uridine and uridine-uridine were obtained from incorporations with α-32P-UTP. Adjacent entries in the guanosine-guanosine and uridine-guanosine columns represent results from individual incubations and electrophoretic separations. Since total recovery of radioactivity was variable in different incubations, comparison is valid only between such pairs of entries.

Two important conclusions follow from these data. First, although uridine-guanosine sequences are very rare when poly C is used as template, they appear with a relative frequency which is similar to the composition of the product (see below) when methyl poly C is used as template. This means that the chain

### Table I

<table>
<thead>
<tr>
<th>Template and incubation time</th>
<th>Guo-Guo</th>
<th>Urd-Guo</th>
<th>Ratio, Urd-Guo to Guo-Guo</th>
<th>Guo-Urd</th>
<th>Urd-Urd</th>
<th>Ratio, Urd-Urd to Guo-Urd</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td>dpm</td>
<td>dpm</td>
<td>%</td>
<td>dpm</td>
<td>dpm</td>
<td>%</td>
</tr>
<tr>
<td>Poly C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>18,075</td>
<td>103</td>
<td>0.6</td>
<td>26</td>
<td>12</td>
<td>46</td>
</tr>
<tr>
<td>60</td>
<td>17,173</td>
<td>110</td>
<td>0.6</td>
<td>20</td>
<td>15</td>
<td>75</td>
</tr>
<tr>
<td>70</td>
<td>13,208</td>
<td>34</td>
<td>0.3</td>
<td>60</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td></td>
<td>0.5</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>6.3% methyl poly C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>10,854</td>
<td>1,284</td>
<td>11.8</td>
<td>226</td>
<td>13</td>
<td>5.7</td>
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<tr>
<td>60</td>
<td>13,430</td>
<td>902</td>
<td>0.8</td>
<td>190</td>
<td>7</td>
<td>3.7</td>
</tr>
<tr>
<td>70</td>
<td>8,614</td>
<td>416</td>
<td>4.8</td>
<td>358</td>
<td>36</td>
<td>10.0</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td></td>
<td>7.8</td>
<td>6.5</td>
<td></td>
</tr>
</tbody>
</table>
TABLE II
Dependence of polymer composition on methylation of template

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Guanosine</th>
<th>Uridine</th>
<th>Ratio, uridine to guanosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly C</td>
<td>13,600</td>
<td>65</td>
<td>0.47</td>
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<tr>
<td>Average</td>
<td>3,830</td>
<td>23</td>
<td>0.60</td>
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<tr>
<td>3.5% methyl C</td>
<td>10,300</td>
<td>282</td>
<td>2.7</td>
</tr>
<tr>
<td>Average</td>
<td>4,940</td>
<td>117</td>
<td>2.4</td>
</tr>
<tr>
<td>6.3% methyl C</td>
<td>2,700</td>
<td>112</td>
<td>4.1</td>
</tr>
<tr>
<td>Average</td>
<td>7,200</td>
<td>295</td>
<td>4.1</td>
</tr>
<tr>
<td>9.0% methyl C</td>
<td>8,700</td>
<td>301</td>
<td>3.5</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

often continues to grow by adding a GDP unit after a UMP unit is incorporated into a polymer formed with methyl poly C template. Second, although the total UMP incorporation is very low with poly C as template, uridine-uridine sequences are nearly as frequent as guanosine-uridine sequences in this case. In marked contrast, the relative frequency of uridine-uridine sequences in polymer made with a methyl poly C template is much lower, at about the level that would be predicted from the average composition of the product. Thus, the possibility of simultaneous synthesis of polyuridylic acid sequence is ruled out and we can conclude that a true copolymer is formed when methyl poly C is used as template.

It was important to show that the composition of newly synthesized copolymer reflected the degree of methylation of the template. Incorporations were performed in which both UTP and GTP were labeled with 14C of equal specific activities, and the composition of the product was determined when poly C and two different preparations of methyl poly C were used as template.

The results of such experiments are shown in Table II. Again, ratios of counts are tabulated for valid comparison between different incubations. The low ratio of uridine to guanosine found in the presence of poly C template might represent some residual formation of polyuridylic acid sequences, as well as some expected background radioactivity in the UMP area. The larger amounts of UTP incorporated in the presence of methyl poly C, however, reflect the composition of the template. Thus, the more highly methylated poly C leads to the formation of a copolymer of uridine and guanosine containing relatively more UMP units. Such a result would suggest that UMP units are inserted opposite the methylated cytosine residues as the methyl poly C is copied. The fact that the resulting copolymer contains less uridine than the methylcytidine content of the template probably indicates a slightly lower efficiency for the incorporation of UTP.

Finally, to test the specificity of this incorporation, other incubations were performed which were similar to those in Fig. 2, except that 14C-labeled ATP or CTP was added to the unlabeled GTP. Although no significant ATP incorporation was observed, some CTP incorporation resulted when either methylated or unmethylated poly C was used as template. We assume that the CTP incorporation reflects a secondary use of newly synthesized guanylyl acid in the polymerase reaction, and is unrelated to the presence of methylcytidine residues in any of the other templates. Thus, methylcytidine residues probably lead specifically to the incorporation of UTP.

DISCUSSION

It seems clear from these data that RNA polymerase will produce a copolymer of uridine and guanosine in the presence of a methylated poly C template. It is more difficult to suggest a base pairing model which would explain this result, however. Not only is one of the conventional hydrogen bonding sites, position 3, occupied by a methyl group in 3-methylcytidylic acid, but the base is protonated at pH 7. It may be that one or more extra-nucleolar hydrogen atoms in the methylated base pairs with the oxygen in uracil, but we have no direct evidence for this as yet.

In addition to simple base pairing considerations, the changes in secondary structure which accompany methylation (7) may be important. The introduction of a positive charge in the template in the region of the methylated base might alter local structure sufficiently to allow incorporation of a noncomplementary nucleotide. The apparent specificity of UTP incorporation in the presence of methylated poly C template suggests a fairly specific mechanism, however.

It is interesting to speculate on the possible mutagenic effects which might occur if similar alklylation and mispairing occurred in vivo. The net result of such a process would be to cause the insertion of uridine in the place of guanosine during transcription or translation. Additional experiments are in progress to determine whether alklylation of other bases results in other examples of mispairing.

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Ribonucleic Acid Polymerase Reactions with Methylated Polycytidylic Acid Templates
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