Altered Specificity of Transfer-Ribonucleic Acid Nucleotidyltransferase in the Presence of Manganese

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1. s-RNA nucleotidyltransferase incorporated CMP into phosphodiesterase-treated s-RNA more rapidly in the presence of Mg^{2+} (10 mM) than in the presence of Mn^{2+} (2 mM). UMP was incorporated more rapidly in the presence of Mn^{2+} , and at high ionic strength the incorporation of CMP was also more rapid in the presence of Mn^{2+} . 2. The capacity of phosphodiesterase-treated s-RNA for CMP, UMP and AMP was increased in the presence of Mn^{2+} . Terminal sequences of more than one UMP or AMP residue were synthesized, but these atypical reactions were inhibited when CTP was added. CMP was incorporated rapidly to form pCpC terminal sequences and then more slowly as longer chains were formed, but very little CMP was incorporated into s-RNA-pCpCpA. 3. CMP was incorporated into phosphodiesterase-treated 5s RNA and ribosomal RNA to form short chains of polyC attached to the primer RNA. This reaction was inhibited by the presence of s-RNA. 4. A small Mn^{2+} -dependent incorporation of CMP was also primed by poly(A). (U) and poly(C). (I).

Rat liver s-RNA nucleotidyltransferase (EC 2.7.7.25) synthesizes the specific terminal pCpCpA sequence of s-RNA* in the presence of Mg^{2+} (Hecht, Zamecnik, Stephenson & Scott, 1958) and can also incorporate single residues of UMP and AMP into positions normally occupied by CMP residues (Daniel & Littauer, 1963; Klemperer & Canellakis, 1966). It is doubtful whether the synthesis of these atypical terminal sequences has physiological significance.

The work now reported arose out of a study of the action of enzymes which attach nucleotides to the 3'-ends of RNA molecules, using as a primer the 5s RNA ('transfer-like' RNA) which is found associated with liver ribosomes (Jackson, 1966). Several polyribonucleotides were found to serve as primers for s-RNA nucleotidyltransferase in the presence of Mn^{2+} , and the same conditions favoured the synthesis of atypical terminal sequences on s-RNA.

* Abbreviations: s-RNA, 'transfer' RNA prepared by phenol extraction from the 105 000g supernatant of rat liver; s-RNA-pCpCpA, s-RNA bearing the terminal 5'-O-phosphorylcytidyl-(3'-5')-cytidyl-(3'-5')-adenosine sequence; s-RNA-pCpC, the same but without the terminal adenylate residue; polyA, polyadenylic acid; polyC, polycytidylic acid; polyI, polyinosinic acid; polyU, polyuridylic acid; poly(A). (U), the double-stranded complex formed by hydrogen bonding between equimolar amounts of polyA and polyU; poly(C). (I), the same for polyC and polyI.

MATERIALS AND METHODS

Materials. [8- ^{14}C]ATP was purchased from The Radiochemical Centre (Amersham, Bucks.), and [6- ^{14}C]CTP, [8- ^{14}C]GTP and [6- ^{14}C]UTP were from Schwartz Bio Research Inc. (Orangeburg, N.Y., U.S.A.). The specific activities (counts/min./ μ mole) of these materials under the same liquid-scintillation conditions (background 35 counts/min., efficiency approx. 75%) as were always used for counting the acid-precipitable reaction product (see below) were taken to be: ATP, 6.0×10^6 ; CTP, 5.3×10^6 ; GTP, 6.0×10^6 ; UTP, 5.2×10^6 . These values were obtained indirectly by multiplying the specific activities obtained after counting at zero thickness on stainless-steel planchets in a windowless gas-flow counter (background 16 counts/min., efficiency approx. 40%) by the factor 1.7. This factor was determined experimentally by counting samples of the acid-precipitable reaction product at zero thickness on glass-paper disks in both the gas-flow and the liquid-scintillation counter. Unlike the precipitated reaction product, radioactive triphosphate samples dried on glass-paper disks did not remain on the surface of the disks and gave falsely low specific activity values by liquid-scintillation counting (which would tend to over-estimate the incorporation of nucleotides into the reaction product).

Enzymes. s-RNA nucleotidyltransferase (EC 2.7.7.25) was prepared from rat liver as described by Canellakis & Herbert (1960). Snake-venom phosphodiesterase (EC 3.1.4.1) was purified from *Crotalus adamanteus* venom (Sigma Chemical Co., London, S.W. 6), by the method of Koerner & Sinsheimer (1957) and had an activity of 4000 units/ml.

Rat liver s-RNA. s-RNA was extracted with phenol from the 105 000g supernatant of rat liver homogenate by the method of Klemperer (1963a). A solution (10 ml.) containing

1.2 mg. of RNA/ml. in 1.0 M-NaCl was applied to a column (180 cm. $\times$ 2 cm.) of Sephadex G-100 which had previously been washed with 1.0 M-NaCl. The column was developed with the same medium at a flow rate of 10 ml./hr. and the s-RNA was collected as a single peak in the region of approx. 0.6–0.75 column volumes of effluent. This s-RNA was precipitated with 2.5 vol. of ethanol at -20° , redissolved in 0.02 M-tris-HCl buffer, pH 7.4, and dialysed against the same buffer. The product was capable of accepting 0.9 μ mole of CMP and 3.8 μ moles of AMP/mg. when incubated with s-RNA nucleotidyltransferase in the presence of Mg^{2+} . This indicates that little degradation of the -pCpCpA terminal had occurred during the preparation.

Rat liver 5s RNA. The preparation from rat liver was based on the methods previously applied by Rosset, Monier & Julien (1964) and Schleich & Goldstein (1966) to *Escherichia coli*, by Galibert, Larsen, Lelong & Boiron (1965) to carcinoma cells and by Jackson (1966) to rat liver. Liver (180 g.) from rats which had been previously starved for 24 hr. was homogenized at 0° with 2.5 vol. of 0.35 M-sucrose-4 mM-MgCl₂-0.02 M-tris-HCl buffer, pH 7.4. The homogenate was centrifuged for 10 min. at 10000g. The supernatant was taken and centrifuged for 90 min. at 105000g, and the resulting microsomal pellet was resuspended at 0° in 400 ml. of 0.05 M-tris-HCl buffer, pH 8.0. The suspension was stirred for 60 min. at 20° with an equal volume of phenol, which had previously been equilibrated with the same buffer. The emulsion was centrifuged for 30 min. at 2000g and the aqueous supernatant was extracted twice more with phenol. The final supernatant was mixed with 0.05 vol. of 1.0 M-potassium acetate, pH 5.5, and 2.5 vol. of ethanol at -20° was added. After 16 hr. at -20° the precipitate of RNA was collected by centrifugation (15 min. at 2000g), dissolved in 100 ml. of 0.05 M-tris-HCl buffer, pH 8.0, containing 0.01% (w/v) of sodium dodecyl sulphate, and the solution extracted twice with phenol as before to remove traces of protein. The RNA was then precipitated with ethanol as before, dissolved in 30 ml. of 0.05 M-tris-HCl buffer, pH 7.4, and dialysed overnight against 1.0 M-NaCl-0.02 M-tris-HCl buffer, pH 7.4. The precipitate of ribosomal RNA was removed by centrifugation (10 min. at 10000g) and the pellet was washed once with 10 ml. of 1.0 M-NaCl and saved for further purification (see below). The RNA in the combined supernatants was precipitated with ethanol as before, dissolved in 10 ml. of 0.02 M-tris-HCl buffer, pH 7.4, and dialysed against the same buffer.

The solution (12 ml. containing 1.5 mg. of RNA/ml.) was applied to a column (3 cm. $\times$ 1 cm.) of DEAE-cellulose powder (Whatman DE 50; Reeve Angel and Co., London, E.C. 4) that had previously been washed with 0.02 M-tris-HCl buffer, pH 7.4. The column was washed with 30 ml. of 0.3 M-NaCl, and then s-RNA and 5s RNA were eluted together in 12 ml. of 1.0 M-NaCl. This eluate was fractionated on a column (180 cm. $\times$ 2 cm.) of Sephadex G-100 as described above for s-RNA. Three successive peaks of RNA were eluted in a profile similar to that described by Galibert *et al.* (1965) and by Schleich & Goldstein (1966). According to these authors the peaks are, in succession, ribosomal RNA, 5s RNA and s-RNA.

The RNA from the third peak was collected by ethanol precipitation and characterized as s-RNA by the fact that after treatment with snake-venom phosphodiesterase (see below) this RNA accepted CMP and AMP in the ratio approx. 2:1 when incubated with s-RNA nucleotidyl-

transferase. The 5s RNA emerged immediately before the s-RNA, and tubes which might have contained any traces of the latter were discarded. The 5s RNA (approx. 4 mg.) was collected by ethanol precipitation, dissolved in 4.0 ml. of 0.02 M-tris-HCl buffer, pH 7.4, and dialysed against the same buffer. Phosphodiesterase treatment showed this RNA to be distinct from s-RNA. In some cases the fractionation on Sephadex G-100 was repeated to remove any traces of s-RNA, but this treatment did not alter the behaviour of the RNA as a primer for s-RNA nucleotidyltransferase.

Rat liver ribosomal RNA. The precipitate collected after dialysis of microsomal RNA against 1.0 M-NaCl was dissolved in 0.05 M-NaCl and was applied to a column (180 cm. $\times$ 2 cm.) of Sephadex G-100 which had been previously washed with 0.05 M-NaCl. The ribosomal RNA was eluted in the same medium and emerged as a single peak which followed the dead space. This RNA was collected by ethanol precipitation, dissolved in 0.02 M-tris-HCl buffer, pH 7.4, and dialysed against the same buffer.

Phosphodiesterase-treated RNA. The concentration of RNA was measured by using an extinction coefficient of 24.8 (260 $m\mu$, 1 cm. light-path) for a solution of 1 mg./ml. in 0.02 M-tris-HCl buffer, pH 7.4 (Hecht *et al.* 1958). RNA samples were treated with snake-venom phosphodiesterase by a procedure based on that of Herbert & Canellakis (1961). Phosphodiesterase (100 units/mg. of RNA) was incubated with RNA (approx. 1 mg./ml. in 0.01 M-MgCl₂-0.1 M-tris-HCl buffer, pH 8.3) for 12 min. at 37° . The reaction was terminated by heating for 90 sec. at 100° , and the product was used directly as a primer for nucleotide incorporation. s-RNA treated in this way is capable of accepting approx. 39 μ moles of CMP and 20 μ moles of AMP/mg. when incubated with s-RNA nucleotidyltransferase in the presence of Mg^{2+} .

The action of phosphodiesterase on s-RNA and on 5s RNA was characterized by measuring the absorption spectra of the released nucleotides after removing undigested RNA with DEAE-cellulose. The reaction product (in 1 ml.) was mixed with 0.02 ml. of 5 M-NaCl and was passed over a column (1 cm. $\times$ 0.5 cm.) of DEAE-cellulose powder which had previously been equilibrated with 0.1 M-NaCl-0.1 M-tris-HCl buffer, pH 8.3. The column was washed with 0.8 ml. of 0.2 M-NaCl and the combined effluents were mixed with 0.2 ml. of 2 N-HCl for measurement of the absorption spectrum in 1 cm. or 4 cm. quartz cells. The observed spectra were matched with curves calculated for mixtures of 5'-mononucleotides, by using published data (Pabst Laboratories, 1956). Fig. 1 (lower curve) shows that 1 mg. of s-RNA (equivalent to 47.6 μ moles, taking molecular weight to be 21000) gave rise to approximately the expected amounts of AMP (42 μ moles) and CMP (72 μ moles). 5s RNA from *E. coli* has a UMP residue at the 3'-terminus and is reported to give rise also to AMP and CMP after extensive digestion with snake-venom phosphodiesterase (Rosset *et al.* 1964). From Fig. 1 it appears that 0.9 mg. of 5s RNA from rat liver (equivalent to 30 μ moles, assuming molecular weight to be 30000) gave rise mainly to UMP (36 μ moles) and to smaller amounts of AMP (11 μ moles) and CMP (12 μ moles).

s-RNA-pCpC and s-RNA-pCpCpA. To prepare s-RNA-pCpC, 200 μ g. of phosphodiesterase-treated s-RNA was incubated in a final volume of 1 ml. with s-RNA nucleotidyltransferase (400 μ g. of protein), 60 μ moles of CTP, 10

Fig. 1. Absorption spectra of nucleotides released by phosphodiesterase. Spectra of nucleotides (in 2.0 ml. of 0.1 N-HCl) from 1 mg. of s-RNA, 1 cm. light-path (●), and from 0.9 mg. of 5s RNA, 4 cm. light-path (○). The calculated absorption spectra under similar conditions are: lower line, 42 μ moles of 5'-AMP + 72 μ moles of 5'-CMP; upper line, 36 μ moles of 5'-UMP + 11 μ moles of 5'-AMP + 12 μ moles of 5'-CMP. The composition of each mixture was calculated by simultaneous equations from the observed extinctions at wavelengths between 260 and 280 $m\mu$ (e.g. Umbreit, Burris & Stauffer, 1957). The extinctions of the mixture at wavelengths from 240 to 300 $m\mu$ were then calculated from the corresponding extinction coefficients of the component 5'-mononucleotides.

μ moles of $MgCl_2$ and 100 μ moles of tris-HCl buffer, pH 8.0, for 40 min. at 37°. Under these conditions the s-RNA became saturated with -pCpC terminal groups. At the end of the incubation the whole reaction mixture was passed over a column (20 cm. $\times$ 1 cm.) of Sephadex G-25 which had been previously equilibrated with 0.02 M-tris-HCl buffer, pH 8.0. The RNA together with protein was recovered quantitatively in the first 2 ml. of eluate after the dead space, and was used directly as a primer. Where s-RNA-pCpCpA was prepared the reaction mixture was the same but 60 μ moles of ATP were included and the substrate was native s-RNA.

Other polynucleotides. PolyA, polyC, polyI and polyU were obtained from Miles Laboratories (Clifton, N.J., U.S.A.), and were dissolved in 0.02 M-tris-HCl buffer, pH 7.4. The concentration of each polynucleotide was calculated in terms of 2'(3')-mononucleotide by measuring the extinction at 260 $m\mu$ (Beaven, Holiday & Johnson, 1955) in 0.1 N-HCl after hydrolysis for 16 hr. at 37° in 0.3 N-KOH.

Calf-thymus DNA from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.) was dissolved at 1 mg./ml. in 0.02 M-tris-HCl (pH 7.4). Denatured DNA was prepared by heating this solution for 30 sec. at 100° and then cooling rapidly in ice.

Incubation for incorporation of [^{14}C]ribonucleotide into RNA. The standard reaction mixture contained (in 0.5 ml.) 10 μ g. of RNA, s-RNA nucleotidyltransferase (60 μ g. of protein), 30 μ moles of [^{14}C]ribonucleoside triphosphate, 50 μ moles of tris-HCl buffer, pH 8.0, and either 5 μ moles of $MgCl_2$ or 1 μ mol of $MnCl_2$. After incubation for 40 min. at 37° the tubes were cooled in ice and 0.2 ml. of 0.2 M-sodium pyrophosphate buffer, pH 7.5, was added to discharge non-enzymically bound ribonucleotides (Kammen, Klemperer & Canellakis, 1961). The acid-insoluble radioactivity was precipitated with 0.3 ml. of 50% (w/v) trichloroacetic acid in the presence of 100 μ g. of ribosomal RNA and 200 μ g. of bovine serum albumin. After standing for 30 min. at 0° the precipitate was collected quantitatively by filtration (Josse & Kornberg, 1962) on 2.5 cm.-diam. glass-paper disks (Whatman GF/C) and washed on the filter three times with 5 ml. quantities of 6% (w/v) trichloroacetic acid at 0°. The disks were dried for 16 hr. in an oven at 37° and then placed in vials together with 10 ml. of scintillation fluid [toluene containing 4 g. of 2,5-diphenyloxazole and 0.05 g. of 1,4-bis-(5-phenyloxazol-2-yl)benzene/l.] for measurement of radioactivity in a liquid-scintillation counter (background 35 counts/min.; efficiency approx. 75%).

Location of [^{14}C]ribonucleotide in reaction product. At the end of the incubation 0.2 ml. of 0.2 M-sodium pyrophosphate and 8 ml. of 0.4 N- $HClO_4$ were added to the reaction mixture. The acid-insoluble reaction product was collected by centrifugation (10 min. at 2000g) and was washed three times by resuspension in 8 ml. of 0.4 N- $HClO_4$ followed by centrifugation. The precipitate was hydrolysed in 0.3 N-KOH for 16 hr. at 37°. The ribonucleosides and 2'(3')-monoribonucleotides that were formed under these conditions were separated in the presence of non-radioactive carrier compounds on 2.5 cm.-wide strips of DEAE-cellulose paper (Falaschi, Adler & Khorana, 1963) by descending chromatography for 3 hr. in 0.2 M-ammonium formate, pH 6.5. The positions of the nucleosides and nucleotides were located by their absorption under a u.v. light. These regions were divided into 2.5 cm. $\times$ 2.5 cm. areas, which were placed in scintillation fluid for measurement of radioactivity in the same way as described above for glass-paper disks. Radioactivity applied to the paper was recovered quantitatively in the nucleoside and nucleotide areas. The method gave results which were reproducible and identical with the results obtained when the same digest was fractionated on columns (1 cm. $\times$ 3 cm.) of Dowex 1 (formate form) as described by Hecht *et al.* (1958). The latter method was employed when ribosomal RNA or 5s RNA was the primer, to allow a determination of radioactivity in the cytidine 2'(3'),5'-diphosphate fraction.

RESULTS

Effect of Mn^{2+} on the incorporation of nucleotides into s-RNA. Nucleotides are known to be incorporated into specific sites by s-RNA nucleotidyltransferase in the presence of Mg^{2+} , and the reaction proceeds at a diminishing rate as the available sites

on the primer become filled. This is illustrated in Fig. 2 (top curve) by the incorporation of CMP into phosphodiesterase-treated s-RNA: after 40 min. the reaction was nearly complete, and the amount of CMP incorporated approached the total capacity of the primer for CMP. The incorporation of any nucleotide may therefore be characterized both by the initial rate and by the total amount of nucleotide that can be incorporated, which in turn reflects the product that is formed.

Although the main interest was in the nature of the products formed in the Mn^{2+} -dependent reactions, a brief comparison was made of the effects of Mg^{2+} (10 mM) and Mn^{2+} (2 mM) on the progress of CMP and UMP incorporation into phosphodiesterase-treated s-RNA (Fig. 2). No attempt was made to define the optimum concentration of either metal ion under any particular set of conditions, and it may also be noted that the nucleoside triphosphate concentration (60 μM) was well below the K_m values (e.g. 180 μM for CTP) found in the presence of Mg^{2+} (Daniel & Littauer, 1963). Under the conditions shown in Fig. 2, CMP was incorporated more rapidly in the presence of Mg^{2+} , but for UMP the reaction was faster in the presence of Mn^{2+} . When the ionic strength was raised by adding ammonium sulphate or sodium chloride, the effect

of the metal ions on CMP incorporation was reversed. The reaction in Mn^{2+} was in fact stimulated by the addition, e.g., of 0.2 M-ammonium sulphate (approx. 5% saturation). In contrast, other experiments (not shown) demonstrated that ammonium sulphate under the same conditions inhibited both the Mn^{2+} - and the Mg^{2+} -dependent incorporation of UMP.

With larger amounts of enzyme than in Fig. 2 the incorporation of all nucleotides in the presence of Mg^{2+} was completed within 40 min. Thus the values shown in Table 1 represent the total capacity of the phosphodiesterase-treated s-RNA for each nucleotide in the presence of Mg^{2+} . The total incorporation of UMP and of AMP was about one-third of that of CMP, but as shown previously (Daniel & Littauer, 1963; Klemperer & Canellakis, 1966) the presence of CTP almost abolished the incorporation of UMP and increased the uptake of AMP, since under these conditions the normal -pCpCpA terminal sequence is synthesized. When Mg^{2+} was replaced by Mn^{2+} the capacity for UMP and AMP was increased. Under these conditions the values after incubation for 40 min. shown in Table 1 do not represent a maximum capacity since incubation for 80 min. gave values approx. 20% higher for CMP, UMP and AMP. CTP inhibited the incorporation of UMP and reduced the incorporation of AMP to about the level found when the -pCpCpA sequence was synthesized in the presence of Mg^{2+} . Therefore when sequences of cytidylate residues could be formed the atypical incorporation of UMP and AMP was prevented.

This conclusion is supported by the data in Table 2. s-RNA-pCpC took up UMP only in traces and

Fig. 2. Incorporation of nucleotides into phosphodiesterase-treated s-RNA. Tubes contained (in 0.5 ml.) 10 μg . of phosphodiesterase-treated s-RNA, 50 $\mu moles$ of tris-HCl buffer, pH 8.0, 30 $m\mu moles$ of [^{14}C]CTP or [^{14}C]UTP as shown and either 5 $\mu moles$ of $MgCl_2$ (○) or 1 $\mu mole$ of $MnCl_2$ (●). s-RNA nucleotidyltransferase was 15 μg . for CTP and 30 μg . for UTP incorporation. $(NH_4)_2SO_4$ (bottom curves) was added to final concn. 0.2 M.

Table 1. Incorporation of nucleotides into phosphodiesterase-treated s-RNA

Tubes contained (in 0.5 ml.) 10 μg . of phosphodiesterase-treated s-RNA, 60 μg . of s-RNA nucleotidyltransferase, 50 $\mu moles$ of tris-HCl buffer (pH 8.0), 30 $m\mu moles$ of ^{14}C -labelled and unlabelled ribonucleoside triphosphates as indicated, and either 5 $\mu moles$ of $MgCl_2$ or 1 $\mu mole$ of $MnCl_2$. The incorporation of radioactivity into acid-insoluble material was measured after incubating for 40 min. at 37° as described in the Materials and Methods section.

Radioactive precursor	Addition	[^{14}C]Ribonucleotide incorporated ($\mu moles/40$ min.)	
		Mg^{2+}	Mn^{2+}
[^{14}C]CTP	—	401	420
[^{14}C]UTP	—	147	243
[^{14}C]UTP	CTP	4	30
[^{14}C]ATP	—	132	332
[^{14}C]ATP	CTP	199	176
[^{14}C]GTP	—	< 1	2

Table 2. Incorporation of nucleotides into s-RNA-pCpC and s-RNA-pCpCpA

The incubations and analyses were similar to those described in Table 1 except that s-RNA-pCpC or s-RNA-pCpCpA replaced phosphodiesterase-treated s-RNA.

$[^{14}C]$ Ribonucleoside triphosphate precursor	$[^{14}C]$ Ribonucleotide incorporated (μ moles/40 min.)			
	s-RNA-pCpC		s-RNA-pCpCpA	
	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}
CTP	25	132	10	37
ATP	195	218	4	7
UTP	—	17	—	3
GTP	—	< 1	—	2

AMP in about the amount expected for the completion of the -pCpCpA terminal, but considerable amounts of CMP were still incorporated in the presence of Mn^{2+} . In contrast very little incorporation of any nucleotide occurred with s-RNA-pCpCpA in the presence of Mg^{2+} or Mn^{2+} . Therefore the presence of the -pCpCpA terminal prevented the additional Mn^{2+} -dependent incorporation of CMP, suggesting that this incorporation occurred by extension of the -pCpC sequence.

That CMP was incorporated by two types of reaction is also suggested by the data in Fig. 3. Thus CMP was rapidly incorporated to an extent that was about equal to the capacity in Mg^{2+} , but further reaction was appreciably slower, even when more enzyme was added at 40 min. However, a further addition of phosphodiesterase-treated s-RNA equal to the amount present initially caused a rapid incorporation of CMP to about twice the amount attained in the first 40 min. The result suggests that the product of the rapid phase of the Mn^{2+} -dependent reaction may be similar to that formed during the Mg^{2+} -dependent incorporation of CMP.

Location of $[^{14}C]$ nucleotides incorporated into s-RNA. The products of these reactions were characterized by measuring the distribution of radioactivity between the nucleoside and the 2'(3')-mononucleotide produced by alkaline hydrolysis (Table 3). Incubation with $[^{14}C]$ CTP in the presence of Mg^{2+} gave a product from which $[^{14}C]$ cytidine and 2'(3')- $[^{14}C]$ CMP were recovered in approximately equal amounts, whereas $[^{14}C]$ ATP in the presence of non-radioactive CTP led to the incorporation of single terminal AMP residues which were recovered as $[^{14}C]$ adenosine (Table 3, lines 1 and 2). These findings reflect the synthesis of the typical -pCpCpA terminal sequences. In the presence of Mn^{2+} , $[^{14}C]$ CTP gave after 40 min. a product which resembled that formed in the

Fig. 3. Effect of further addition of phosphodiesterase-treated s-RNA on the Mn^{2+} -dependent incorporation of CMP. The incorporation of $[^{14}C]$ CMP into phosphodiesterase-treated s-RNA in the presence of Mn^{2+} was measured under the same conditions as in Table 1 for 20 and 40 min. (□). After 40 min. certain tubes received a further addition of either 60 μ g. of enzyme (○) or 10 μ g. of phosphodiesterase-treated s-RNA (●).

presence of Mg^{2+} , but after 80 min. longer chains (average 2.5 residues of CMP) were formed (Table 3, lines 3 and 4). Thus Mn^{2+} favoured extension of the -pCpC sequence. Table 3 (lines 5 and 6) also shows the tendency of UMP and AMP (which are known to be incorporated only as single terminal residues in the presence of Mg^{2+}) to form short terminal chains in the presence of Mn^{2+} , since an appreciable proportion of the radioactivity was recovered in the 2'(3')-ribonucleotide fraction.

CMP incorporation into ribosomal RNA and 5s RNA. In the presence of Mg^{2+} only traces of nucleotides were incorporated into native or phosphodiesterase-treated ribosomal RNA and 5s RNA, but Table 4 shows that in the presence of Mn^{2+} these nucleic acids were primers for CMP incorporation, especially when they had previously been treated with phosphodiesterase. Small amounts of AMP and UMP were also incorporated into phosphodiesterase-treated 5s RNA. The presence of small amounts of native s-RNA readily inhibited the incorporation of CMP into the atypical primers. In other experiments (not shown) it was also found that the presence of 0.2M-ammonium sulphate inhibited the Mn^{2+} -dependent incorporation of CMP into the phosphodiesterase-treated primers by more than 90%.

The behaviour of 5s RNA differed in one respect from that of ribosomal RNA. Thus controls to phosphodiesterase-treated ribosomal RNA (like

Table 3. *Location of ribonucleotides incorporated into phosphodiesterase-treated s-RNA*

The reaction mixtures were the same as in Table 2 except that the specific activity of all triphosphates was doubled. The reaction product was precipitated and washed with 0.4N-HClO₄, and then hydrolysed for 16 hr. at 37° with 0.3N-KOH as described in Materials and Methods section. A portion of the digest was fractionated on DEAE-cellulose paper after the addition of carrier compounds. The recovery of radioactivity was essentially complete in all cases.

Expt. no.	[¹⁴ C]Ribo-nucleoside triphosphate precursor	Metal ion	Incubation time (min.)	Radioactivity recovered (%)		Total radioactivity (counts/min.)	Chain length
				In ribo-nucleoside	In ribo-nucleotide		
1	CTP	Mg ²⁺	40	56	44	1780	1.8
2	ATP*	Mg ²⁺	40	98	2	650	1.0
3	CTP	Mn ²⁺	40	52	48	1620	1.9
4	CTP	Mn ²⁺	80	40	60	1130	2.5
5	UTP	Mn ²⁺	40	77	23	940	1.3
6	ATP	Mn ²⁺	40	53	47	1230	1.9

* Non-radioactive CTP (30 mμmoles) was also added to this tube.

Table 4. *Incorporation of nucleotides into ribosomal RNA and 5s RNA*

Tubes were incubated for 40 min. at 37° and contained (in 0.5 ml.) 30 mμmoles of the [¹⁴C]ribonucleoside triphosphate shown, 60 μg. of s-RNA nucleotidyltransferase, 50 μmoles of tris-HCl buffer (pH 8.0), 1 μmole of MnCl₂ and 10 μg. of the primer RNA shown. Heated 5s RNA was heated for 90 sec. at 100° under the same conditions as were used to terminate the treatment with phosphodiesterase. Where indicated, native s-RNA was added in the amount shown; under these conditions 30 μμmoles of [¹⁴C]CMP were incorporated when 10 μg. of native s-RNA alone was the primer.

[¹⁴ C]Ribo-nucleoside triphosphate precursor	Addition	[¹⁴ C]Ribonucleotide incorporated (μμmoles/40 min.)			
		Ribosomal RNA	Phosphodiesterase-treated ribosomal RNA	5s RNA	Heated 5s RNA
CTP	—	83	188	35	1025
ATP	—	3	11	5	32
UTP	—	2	3	2	31
GTP	—	1	2	<1	<1
CTP	s-RNA (0.2 μg.)	—	115	—	540
CTP	s-RNA (1.0 μg.)	—	66	—	244
CTP	s-RNA (10.0 μg.)	—	36	—	52

those to phosphodiesterase-treated s-RNA) which were heated for 90 sec. at 100° in the presence of 0.01 M-magnesium chloride-0.1 M-tris-hydrochloric acid buffer, pH 8.3, behaved like the untreated RNA whereas 5s RNA after heating under the same conditions showed a large increase in primer activity for CMP incorporation (Table 4, last column). The cause of this is not understood, but it might reflect a partial degradation of 5s RNA under these conditions, or perhaps a change in tertiary structure as can occur, e.g. with s-RNA in the absence of Mg²⁺ (Lindahl, Adams & Fresco, 1966).

Table 5 shows that the primers prepared from ribosomal RNA and 5s RNA differed from phosphodiesterase-treated s-RNA in that there was no evidence for a rapid reaction with CTP leading to the saturation of certain sites on the RNA. The high levels of incorporation suggest the formation of chains of CMP residues and this is confirmed in Table 6. A high proportion of the radioactivity was

Table 5. *Incorporation of CMP into ribosomal RNA and 5s RNA*

The reaction mixtures were similar to those described in Table 4, except that s-RNA nucleotidyltransferase was 120 μg. and [¹⁴C]CTP was the radioactive precursor in all cases.

Incubation time (min.)	[¹⁴ C]CMP incorporated (μμmoles)		
	Phosphodiesterase-treated ribosomal RNA	Phosphodiesterase-treated 5s RNA	Heated 5s RNA
20	195	930	396
40	450	1380	700
80	740	1950	—

recovered in 2'(3')-[¹⁴C]CMP, indicating the formation of chains of more than 10 residues of CMP. No radioactivity was recovered in cytidine 2'(3'),5'-

Table 6. Location of CMP incorporated into phosphodiesterase-treated ribosomal and 5s RNA

The reaction mixtures were similar to those described in Table 5 and the reaction product was characterized as described in Table 3, except that the fractionation of the digest was carried out on a Dowex 1 (formate form) column. The recovery of radioactivity was essentially complete in all cases.

Phosphodiesterase-treated RNA	Incubation time (min.)	Radioactivity recovered (%)			Total radioactivity (counts/min.)	Chain length
		In cytidine	In CMP	In CDP		
Ribosomal RNA	80	9.0	91.0	<1	2650	11.1
5s RNA	40	15.0	85.0	<1	3695	6.7
5s RNA	80	8.5	91.5	<1	7570	11.8

Table 7. Incorporation of CMP into synthetic polyribonucleotides

Tubes were incubated for 40 min. at 37°, and contained (in 0.5 ml.) 60 μ g. of s-RNA nucleotidyltransferase, 30 m μ -moles of [^{14}C]CTP, 1 μ mole of $MnCl_2$, 50 μ mole of tris-HCl buffer, pH 8.0, and the polymer indicated (25 m μ mole of nucleotide). Poly(A).(U) and Poly(C).(I) consisted of mixtures of the component homopolymers (each 25 m μ -moles): these homopolymers were added separately but it is highly probable that they associated to form the double-stranded complex in each case.

Polyribonucleotide primer	[^{14}C]CMP incorporated (μ mole)
PolyA	1
PolyC	<1
PolyI	1
PolyU	<1
Poly(A).(U)	48
Poly(C).(I)	55

diphosphate, and this shows that the newly formed polyC had no free 5'-end but was attached to the primer RNA.

CMP incorporation under other conditions. Table 7 shows that the homopolymers did not prime CMP incorporation when tested individually, but a small incorporation was observed in the presence of Mn^{2+} when equimolar amounts of polyA and polyU or polyC and polyI were present. Thus double-stranded hydrogen-bonded complexes could act as primers for CMP incorporation. However, none of these polyribonucleotides primed the incorporation of AMP (results not shown).

In other experiments no incorporation of CMP occurred in the absence of added primer, nor was any reaction primed by native DNA or denatured DNA.

DISCUSSION

The results show that in the presence of Mn^{2+} atypical terminal sequences were attached to s-RNA and atypical primers were used as nucleo-

tide acceptors by s-RNA nucleotidyltransferase. The characteristics of the new reactions suggested a close relationship to the normal behaviour of this enzyme and make it unlikely that a separate Mn^{2+} -dependent enzyme was involved. Thus the formation of the atypical terminal sequences on s-RNA was inhibited when the typical -pCpCpA end was formed, and the reactions with unusual primers were readily inhibited by low concentrations of s-RNA.

It is unlikely that the reaction with 5s RNA represents any true specificity for this molecule since the characteristics of the reaction with ribosomal RNA were very similar. The primer activity of 5s RNA and of ribosomal RNA may depend on the existence of internally hydrogen-bonded regions which have some resemblance to s-RNA. In support of this, double-stranded structures like poly(A).(U) had some primer activity, but single-stranded molecules were inactive. Phosphodiesterase, acting in the same way as on s-RNA, would preferentially remove regions which are not hydrogen-bonded from the 3'-ends of 5s RNA and ribosomal RNA, so increasing the resemblance to the form of s-RNA which acts as a primer.

These changes in the specificity of s-RNA nucleotidyltransferase recall certain Mn^{2+} -dependent changes in the properties of other enzymes involved in nucleic acid synthesis. Other examples of atypical products synthesized in the presence of Mn^{2+} are the incorporation by DNA-dependent deoxyribonucleotidyltransferase (EC 2.7.7.7) of ribonucleotides (Berg, Fancher & Chamberlin, 1963), and the synthesis by DNA-primed RNA nucleotidyltransferase (EC 2.7.7.6, 'RNA polymerase') of homopolymers composed of single species of ribonucleotides (e.g. Chamberlin & Berg, 1964; Fox & Weiss, 1964; Stevens, 1964; Ballard & Williams-Ashman, 1966). Again, in the presence of Mn^{2+} the specific viral RNA primer of a virus-induced RNA nucleotidyltransferase ('RNA replicase') can be replaced by other RNA molecules (Haruna & Spiegelman, 1965) and DNA-primed RNA poly-

merase can be primed by polyribonucleotides (e.g. Krakow & Ochoa, 1963; Fox, Robinson, Haselkorn & Weiss, 1964; Gomatos, Krug & Tamm, 1964; Niyogi & Stevens, 1965; Ballard & Williams-Ashman, 1966).

Apart from its effects on the nature of the reaction product, the effects of Mn^{2+} on the rate of the reaction (Fig. 2) may be contrasted with those of Mg^{2+} . Thus CMP was incorporated into s-RNA more rapidly in the presence of Mg^{2+} , but at high ionic strength the rate was faster with Mn^{2+} . This change of ion specificity resembles, at least superficially, that found with mammalian nuclear RNA polymerase in the presence of ammonium sulphate (Widnell & Tata, 1964; Pogo, Allfrey & Mirsky, 1966). The situation was different for UMP incorporation into s-RNA. This reaction resembled the incorporation of CMP into the atypical primers, since both reactions were Mn^{2+} -dependent rather than Mg^{2+} -dependent, and the addition of ammonium sulphate was strongly inhibitory.

The Mn^{2+} -dependent incorporation of CMP into atypical primers, especially phosphodiesterase-treated 5s RNA, could be described as a polyC polymerase reaction, and it raises the question of the relationship of this enzyme reaction to the large group of enzymes synthesizing homopolymers, in particular to those which are Mn^{2+} -primed (e.g. Klemperer, 1963*b*; Page, Haynes & Klemperer, 1966). The latter will not incorporate nucleotides into s-RNA in the presence of Mg^{2+} . However, the present paper again shows that an enzyme of well-defined function can synthesize a homopolymer as an atypical product. The results also emphasize that the existence of a particularly effective primer for a homopolymerase reaction *in vitro* does not necessarily imply that the same primer functions in this reaction *in vivo*.

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