account the present data as well as the data of Kornberg's group on animal, plant, and bacterial DNAs. The only justifiable conclusion that may be drawn, at least in the case of mouse liver mtDNA, is that the frequencies of dinucleotides are decidedly less random in mtDNA than in any other double-stranded DNA species studied thus far.

References

Borst, M., and Kroon, A. M. (1969), *Int. Rev. Cytol. 26*, 107.

Chargaff, E. (1968), *Progr. Nucl. Acid Res. Mol. Biol. 8*, 297.

Chatterjee, S. K., Dass, H. K., and Roy, S. C. (1966), *Biochim. Biophys. Acta 114*, 349.

Cummins, J. E., Rush, H. P., and Evans, T. E. (1967), *J. Mol. Biol. 23*, 281.

Georgatsos, J. G., Antonoglou, O., and Gabrielides, C. (1970), *Arch. Biochem. Biophys. 136*, 219.

Hurwitz, J., Furth, J., Anders, M., and Evans, A. (1962), *J. Biol. Chem. 237*, 3752.

Josse, J., Kaiser, A. D., and Kornberg, J. (1961), *J. Biol. Chem. 236*, 864.

Josse, J., and Swartz, M. (1963), *Methods Enzymol. 6*, 739.

Markham, R., and Smith, J. D. (1952), *in* The Nucleic Acids, Vol. 1, Chargaff, E., and Davidson, J. N., Ed., New York, N. Y., Academic Press, p 267.

Murray, K. (1970), *Biochem. J. 118*, 831.

Stern, H. (1968), *Methods Enzymol. 12B*, 100.

Symons, R. H. (1970a), *Biochim. Biophys. Acta 209*, 296.

Symons, R. H. (1970b), *Biochem. Biophys. Res. Commun. 38*, 807.

Weiss, S. B. (1968), *Methods Enzymol. 12B*, 559.

Weiss, S. B., and Nakamoto, T. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 1400.

Wyatt, G. P. (1951), *Biochem. J. 48*, 584.

Comparison of Some Reactions Catalyzed by Deoxyribonucleic Acid Polymerase from Avian Myeloblastosis Virus, *Escherichia coli*, and *Micrococcus luteus**

Robert D. Wells,† Rolf M. Flügel, Jacquelynn E. Larson, Paul F. Schendel, and Raymond W. Sweet

ABSTRACT: A comparative study was performed on the template specificities of the highly purified DNA polymerases from *Escherichia coli* and *Micrococcus luteus* and of a partially purified DNA polymerase from virions of avian myeloblastosis virus (AMV). The three DNA polymerases show approximately the same capacity to utilize twenty different high molecular weight templates. Thus, when tested with polymer templates (primers), the two bacterial DNA polymerases are at least as effective "reverse transcriptases" as the tumor virus associated DNA polymerase. However, $(rA)_n \cdot$oligo(dT) is a markedly better template than $(dA)_n \cdot$oligo(dT) for the AMV DNA polymerase, as reported previously. For the *M. luteus* DNA polymerase, the two templates are approximately equally effective. The AMV DNA polymerase provides faithful DNA synthesis when either DNAs or RNAs serve as templates (primers). DNA synthesis is dependent on the presence of a suitable primer strand and the newly synthesized DNA strand is covalently attached to the primer strand through a phosphodiester linkage. Thus, when a polyribonucleotide serves as a primer, the new DNA strand is joined to an RNA molecule. The AMV DNA polymerase apparently cannot initiate the synthesis of a new DNA strand. This behavior is identical with that observed for the two bacterial DNA polymerases.

A variety of nucleic acid metabolizing enzymes is associated with virions of RNA tumor viruses. These include DNA polymerase(s) (Baltimore, 1970; Temin and Mizutani, 1970; Spiegelman *et al.*, 1970), DNA ligase (Mizutani *et al.*, 1971), nuclease(s) (Mizutani *et al.*, 1970, 1971), nucleoside diphosphokinase (Miller and Wells, 1971), and other nucleotide kinases and phosphotransferases (Roy and Bishop, 1971; Mizutani and Temin, 1971). Other activities are also present (Mizutani and Temin, 1971).

Studies have indicated that some DNAs, RNAs, and DNA·RNA hybrids will serve as templates (primers) for the virion-associated DNA polymerases (Spiegelman *et al.*, 1970; Riman and Beaudreau, 1970; Fujinaga *et al.*, 1970; Mizutani *et al.*, 1970; Hatanaka *et al.*, 1970; Scolnick *et al.*, 1970, 1971; Duesberg *et al.*, 1971; Baltimore and Smoler, 1971). This and related work has led to the notion that these enzymes catalyze the flow of genetic information from RNA to DNA ("reverse transcription"). However, it is not certain if the "reverse transcriptases" have truly unique activities or have properties similar to well-characterized DNA polymerases.

Hence, a comparison was made of the catalytic capacities of a functionally pure viral polymerase (free of detectable contaminating activities) with two well characterized polymerases. Due to the comparative nature of this work, nucleic acid structural problems were obviated and empirical comparisons can be drawn.

* From the Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin, Madison, Wisconsin 53706. *Received August 23, 1971.* This work was supported by the National Science Foundation (GB-8786), the National Institutes of Health (Contract No. 71-2275), the Damon Runyon Fund (DRG-1049-A), and the Jane Coffin Childs Fund (No. 272). R. W. S. is a National Institutes of Health predoctoral fellow (GM 39327).

† To whom to address correspondence.

Materials and Methods

Substrates and Polynucleotides.[1] Deoxyribonucleoside triphosphates were as described (Burd and Wells, 1970). The oligonucleotide $d(pT)_{13}$ was prepared and characterized as described (Khorana and Connors, 1966). Double-stranded DNA polymers were characterized by analytical buoyant density centrifugation in alkaline solution (Wells and Larson, 1970; Wells *et al.*, 1970). All DNAs used herein were free of detectable contamination (less than 2%) and had density values identical (± 0.001 g/cm^3) with authentic samples. The single-stranded ribopolymers were obtained from Schwarz–Mann, Inc., and were purified by phenol extraction and dialysis (Wells *et al.*, 1967). $(rA\text{-}rU)_n\cdot(rA\text{-}rU)_n$ and $(rG\text{-}rC)_n\cdot(rG\text{-}rC)_n$ were prepared and characterized as described previously (DeClercq *et al.*, 1971). Greater than 50-fold net transcription was observed in both cases. Hence the amount of contamination of the RNA by the template DNA must be less than 2%; this was also shown by analytical density gradient centrifugation.

$(dA)_n$, $(dT)_n$, $(dG)_n$, and $(dC)_n$ were prepared by preparative alkaline CsCl density gradient centrifugation (Wells and Blair, 1967) of the parent duplex polymers, $(dA)_n\cdot(dT)_n$ and $(dG)_n\cdot(dC)_n$ [or $(dI)_n\cdot(dC)_n$], respectively. Although the single-stranded polymers which were prepared by this method contained no detectable contamination (<2%) with the complementary polymer (as judged by analytical alkaline density gradient centrifugation), they may contain trace amounts of complementary oligonucleotides. These may effectively serve as primers in the polymerase reactions when the single-stranded polymer is tested as a template (Harwood and Wells, 1970; R. W. Sweet and R. D. Wells, unpublished results). To obviate this problem with the polypyrimidine DNAs, $(dT)_n$ and $(dC)_n$ were each depurinated with acid (Harwood and Wells, 1970).

$s^0_{20,w}$ values were determined in 0.1 M NaOH–0.9 M NaCl solution (Studier, 1965) for the DNAs and in 0.15 M NaCl–0.015 M sodium citrate solution (pH 7.0) for the RNAs. The single-stranded DNA molecular weights ($\times 10^{-5}$) are $(dA)_n$, 3.5; $(dT)_n$, 3.2; $(dA\text{-}dT)_n$, 5.2; $(dG)_n$, 0.9; $(dC)_n$, 1.9; $(dG\text{-}dC)_n$, 2.7. The RNA sedimentation values are $(rA\text{-}rU)_n\cdot(rA\text{-}rU)_n$, 3.4; $(rA)_n$, 1.9; $(rU)_n$, <1; $(rG\text{-}rC)_n\cdot(rG\text{-}rC)_n$, 4.4; $(rG)_n$, 4.5; $(rC)_n$, <1.

Enzymes. The *E. coli* DNA polymerase I was fraction 6 from the procedure of Jovin *et al.* (1969) and is homogeneous (Burd and Wells, 1970). The *M. luteus* DNA polymerase was the most highly purified fraction in the method of Harwood *et al.* (1970a).

The AMV DNA polymerase was purified approximately 400-fold (20-fold if rATP or a phosphatase inhibitor were included in the assay of the disrupted virus). The purification steps were ether disruption of the virus, sucrose gradient centrifugation, and DEAE-Sephadex column chromatography. At this stage, the enzyme (fraction III) had a specific activity of 400–500. For some experiments, the enzyme was further purified by hydroxylapatite column chromatography (fraction IV). One unit of AMV DNA polymerase activity is defined as 1 mμmole of total nucleotide incorporated per milliliter per hour under conditions similar to those described below where 65 μM $(dA\text{-}dT)_n\cdot(dA\text{-}dT)_n$ served as template.

DNA Polymerase Reactions with Polymer Templates (Primers). For the *M. luteus* and the *E. coli* DNA polymerases, the reactions (0.1 ml) contained: Tris-HCl buffer (pH 8.1), 50 mM; $MgCl_2$, 5 mM; 2-mercaptoethanol, 1 mM; dATP and dTTP each at 1 mM, or dGTP and dCTP each at 1 mM; ^{3}H-labeled deoxytriphosphate, 1–100 $\times 10^6$ cpm/reaction; polynucleotide (as indicated), 100 μM; *M. luteus* DNA polymerase (10 units) or *E. coli* DNA polymerase I (31 units). For the homopolymer duplexes, the individual strands were combined in an equimolar ratio. In all cases, the first three components and the polynucleotides were combined, kept at 60° for 5 min, and then slowly cooled (10 min). The reactions were at 37° and were monitored as previously described (Harwood *et al.*, 1970a). This method of annealing provides no assurance that polymers are in the double-stranded form. For example, $(dA)_n\cdot(rU)_n$ probably exists as a triplex (Riley *et al.*, 1966). However, it was not deemed appropriate to characterize the degree of strandedness for this study, since the incubation mixture itself contained components (divalent metal ions, enzymes) which could promote helix–helix displacement reactions. Instead, each polynucleotide was analyzed in the single-stranded form (see above).

For the AMV DNA polymerase, the reactions (0.1 ml) contained: Tris-HCl buffer (pH 8.3), 50 mM; $MgCl_2$, 2 mM; NaCl, 60 mM; potassium phosphate (pH 8.3), 5 mM; dithiothreitol, 10 mM; dATP and dTTP each at 30 μM or dGTP and dCTP each at 30 μM; [^{3}H]deoxyribotriphosphate, 1–100 $\times 10^6$ cpm/reaction; polynucleotide (as indicated), 50–60 μM; and AMV DNA polymerase (fraction III), 0.7 unit. The first five components and the polynucleotide were combined and kept at 60° for 5 min and then slowly cooled. The reactions were at 41° and were monitored as above.

The unequal extents of incorporation of both dNMPs in some cases, when the alternating dinucleotide polymers served as templates (primers), is due to the fact that individual reactions were performed in separate tubes. Also it is difficult to ascertain unequivocally the absolute specific activity of a tritiated nucleotide due to the variation in efficiency depending on whether the isotope is in a monomer or in a polymer.

DNA Polymerase Reactions with Polymer·Oligomers. Reactions with the *M. luteus* DNA polymerase were as described above except that 250 μM dTTP was the sole substrate, 1 unit of DNA polymerase was added and $(dA)_n$ or $(rA)_n$, and $d(pT)_{13}$ (as indicated) replaced the polynucleotide. Except for omitting the annealing step, the reactions were performed as above.

Reactions with the AMV DNA polymerase were as described above except that dTTP was the sole substrate and $(dA)_n$ or $(rA)_n$ and $d(pT)_{13}$ (as indicated) replaced the polynucleotide.

Covalent Joining of DNA to RNA. The *M. luteus* DNA polymerase reaction (0.50 ml) was as above for polymer templates except that [α-^{32}P]dATP (0.6 mM, 4.6×10^7 cpm) was the sole substrate and 38 μM $(rA)_n$ and 95 μM depurinated $(dT)_n$ replaced the polynucleotide. In the control reaction, $(rA)_n$ was replaced by 42 μM $(dA)_n$.

After 60 min, the reaction was stopped by addition of NaCl (1.0 M final concentration) and NaEDTA (1.5×10^{-2} M); the solution was heated at 75° for 15 min. After phenol extraction, the reaction products were dialyzed as described (Burd and Wells, 1970). The overall recovery was 71%.

Paper chromatography of a sample of both reaction products in solvent A revealed that all the radioactivity remained at the origin. The same result was found when another sample of the reaction products was treated with bacterial alkaline phosphatase (solution was made 1.0 M in Tris-HCl (pH 8.0) and 15 μg of alkaline phosphatase was added and kept at 37°

[1] IUPAC nomenclature is used throughout; *i.e.*, poly(dA) is equivalent to $(dA)_n$.

TABLE I: A and T (or U) Containing DNA and RNA Polymers as Templates (Primers).[a]

Template Polymer	A. *E. coli* DNA Polymerase Nucleotide Incorporation				B. *M. luteus* DNA Polymerase Nucleotide Incorporation				C. AMV DNA Polymerase Nucleotide Incorporation			
	dAMP		dTMP		dAMP		dTMP		dAMP		dTMP	
	Incorp	%	Incorp	%	Incorp	%	Incorp	%	Incorp	%	Incorp	%
dA·dT	250	100	260	100	68	100	30	100	0.92	100	0.10	100
rA·dT	260	104	145	56	81	119	20	66	3.30	359	3.72	3720
dA·rU	110	44	160	62	4	6	13	43	0.055	6	0.034	34
rA·rU	60	24	85	33	2	3	2	7	<0.001	<0.1	0.020	20
(dA-dT)·(dA-dT)	122	49	120	46	120	177	140	466	3.28	357	3.39	3390
(rA-rU)·(rA-rU)	15	6	18	7	10	15	4	13	0.06	6.5	0.14	140
dA	68	27	160	61	4	6	7	23	0.05	5.4	0.05	50
dT	3	1.2	1.5	<1	1.5	2	1.5	2	<0.001	<0.1	<0.001	<1.0
rA	1	<1	1	<1	1	<1	1	<3	<0.001	<0.1	<0.001	<1.0
rU	1	<1	1	<1	1	<1	1	<3	<0.001	<0.1	<0.001	<1.0

[a] The amount of incorporation of deoxyribomonophosphate is in terms of millimicromoles per milliliter in 2 hr. The relative extent of incorporation is expressed as percentage of extent observed for the homopolymeric DNA duplex. All data are taken from multiple-point kinetic plots. Both dATP and dTTP are present in all experiments.

for 2 hr) prior to the chromatography. A standard acid-insolubility assay also demonstrated that all of the isolated product was polymeric.

A portion of the reaction products was made 0.3 M in KOH and the solution was incubated at 37° for 17 hr. The hydrolysate was neutralized at 0° with Dowex 50 (acid form); the resin was washed with a small volume of 50% aqueous ethanol–1% ammonia solution. The combined washings were concentrated and chromatographed in solvent A. Nonradioactive 3′-rAMP was added as a marker before the run. The radioactivity on the chromatogram was analyzed with a Packard radiochromatogram scanner, or alternatively, the paper was cut into 1.0-cm strips and was counted in a liquid scintillation counter.

The AMV DNA polymerase reaction (1.0 ml) was as described above except that [α-^{32}P]dGTP (30 μM, 1.67 × 10^9 cpm) was the substrate and 27 μM depurinated $(dC)_n$ and 27 μM $(rG)_n$ replaced the polynucleotide. The control reaction contained 27 μM $(dG)_n$ instead of $(rG)_n$.

The reaction was stopped after 120 min and worked up as described above. The overall recovery was 62%. After alkaline hydrolysis and desalting with Dowex 50, the reaction products were chromatographed in solvent B and analyzed as described above.

Other Methods. Descending paper chromatography was performed on Whatman 1 paper in the following systems: solvent A, isobutyric acid–concentration NH_3–H_2O (66:1:33, v/v); solvent B, 0.1 M sodium phosphate (pH 6.8) (100 ml)–ammonium sulfate (60 g)–1-propanol (2 ml). Thin-layer chromatography was performed on PEI-cellulose plates (Baker Chemical Co.) which were prewashed with water and air-dried. The chromatography system was 0.75 M KH_2PO_4 (pH 3.5).

Results

DNA, RNA, and DNA·RNA Hybrid Templates (Primers). A variety of polynucleotides was tested as templates (primers) for the DNA polymerase from *E. coli*, *M. luteus*, and AMV. Tables I and II show the results; typical kinetics of DNA synthesis are shown in Figures 1, 2, and 3 to emphasize the differences in reaction rates with different polymers. The results are as follows. (1) Double-stranded DNAs are more active than single-stranded DNAs with all three enzymes. $(dA)_n$·$(dT)_n$, $(dA\text{-}dT)_n$·$(dA\text{-}dT)_n$, $(dG)_n$·$(dC)_n$, and $(dG\text{-}dC)_n$·$(dG\text{-}dC)_n$ are acceptable templates (primers) whereas the $(dA)_n$, $(dT)_n$, $(dG)_n$, and $(dC)_n$ are relatively inactive.

A relatively small amount of incorporation is found with $(dA)_n$ (Table I) and with $(dG)_n$ (Table II). This is due, no doubt, to trace contamination (less than 2%) of these polymers with pieces of the complementary strands. We previously reported (Harwood and Wells, 1970) that less than 0.1% contamination of a single-stranded polymer with short complementary oligomers will give these results.

Single-stranded $(dC)_n$ (Table II) is a poor template; how-

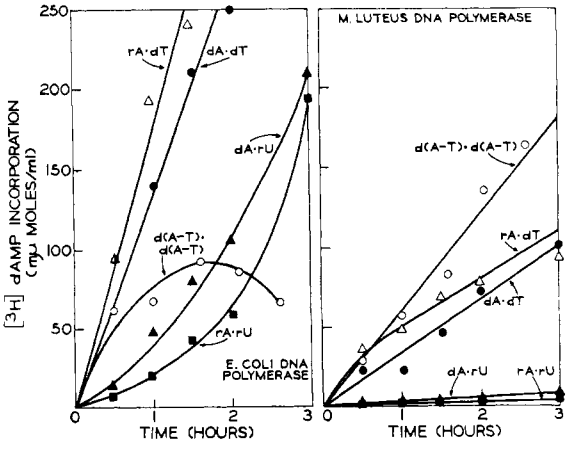

FIGURE 1: Kinetics of polymer synthesis with A and T (or U) containing templates (primers). In each case, the template (primer) is indicated. Other details are given in Materials and Methods.

TABLE II: G and C Containing DNA and RNA Polymers as Templates (Primers).[a]

Polymer Template	A. *E. coli* DNA Polymerase Nucleotide Incorporation				B. *M. luteus* DNA Polymerase Nucleotide Incorporation				C. AMV DNA Polymerase Nucleotide Incorporation			
	dGMP		dCMP		dGMP		dCMP		dGMP		dCMP	
	Incorp	%	Incorp	%	Incorp	%	Incorp	%	Incorp	%	Incorp	%
dG·dC	66	100	25	100	25	100	2.5	100	5.05	100	0.04	100
rG·dC	76	115	20	80	6	24	1	40	5.66	112	0.05	125
dG·rC	2	3	2	8	3	12	2	80	0.91	18	0.35	875
rG·rC	1.5	2	1	4	2	8	1	40	<0.001	<0.02	<0.001	<2.5
(dG-dC)·(dG-dC)	22	33	20	80	16	64	16	640	0.62	12	0.49	1225
(rG-rC)·(rG-rC)	8	12	8	32	8	32	8	320	0.30	6	0.37	925
dG	3	4	3	12	3	12	3	120	0.04	0.8	0.18	450
dC	16	24	7	28	2.5	10	<1.5	<60	<0.001	<0.02	<0.001	<2.5
rG	<1.5	<3	<1.5	<6	<1.5	<6	<1.5	<60	<0.001	<0.02	<0.001	<2.5
rC	<1.5	<3	<1.5	<6	<1.5	<6	<1.5	<60	<0.001	<0.02	<0.001	<2.5

[a] The amount of incorporation of deoxyribomonophosphate is in terms of millimicromoles per milliliter in 2 hr. The relative extent of incorporation is expressed as percentage of extent observed for the homopolymeric DNA duplex. All data are taken from multiple-point kinetic plots. Both dGTP and dCTP are present in all experiments.

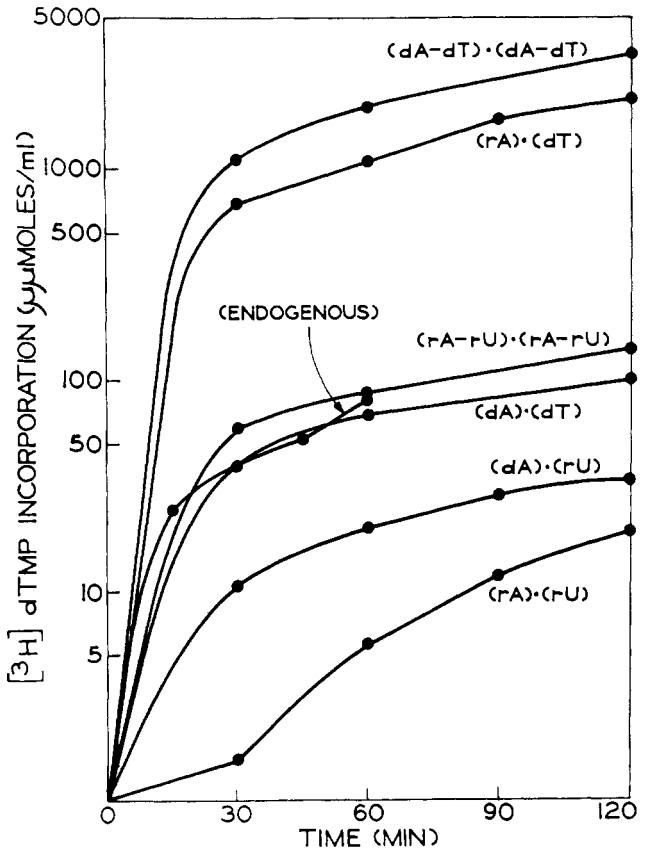

FIGURE 2: Kinetics of nucleic acid synthesis with the AMV DNA polymerase using A and T (or U) containing templates (primers). In each case, the template is indicated beside the final time points. All curves represent the incorporation of [^{3}H]dTMP except in the case of the endogenous reaction (Nonidet P-40 disrupted virions) where the incorporation of [^{3}H]dCMP was monitored. All four dNTP's were present for the endogenous reaction whereas only dATP and dTTP were present in all other reactions. Other details are given in Materials and Methods.

ever, it was unexpected that it is active at all since the polymer preparation included an acid-depurination step (Materials and Methods). That $(dC)_n$ shows any activity is believed due to its unusually high pK (Inman, 1964) which allows it to exist in a partially ordered structure (duplex or triplex). Similar results have been observed with the calf thymus DNA polymerase (F. Bollum, unpublished work).

(2) Double-stranded RNAs are weakly active and single-stranded RNAs are ineffectual as templates (Tables I and II). It should be noted that $(rA)_n \cdot (rU)_n$ is more active with the *E. coli* DNA polymerase than with the other two enzymes (Figures 1 and 2). Lee-Huang and Cavalieri (1963) previously showed that this RNA was active for the synthesis of $(dA)_n \cdot (dT)_n$ by the *E. coli* enzyme. The incorporation of dCMP is relatively high for the *M. luteus* and AMV enzymes with $(rG\text{-}rC)_n \cdot (rG\text{-}rC)_n$ due to the strictly alternating nature of the product DNA. The dGMP incorporation is relatively low in these cases (Table II).

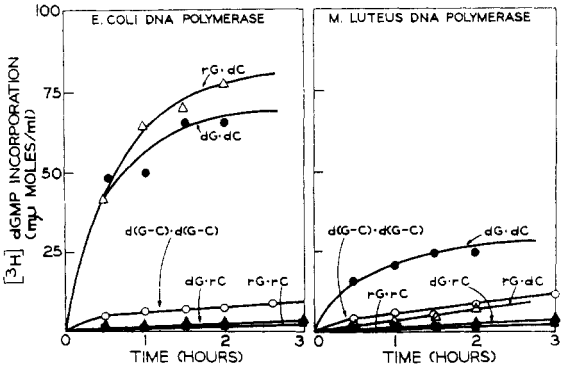

FIGURE 3: Kinetics of polymer synthesis with G and C containing templates (primers). In each case, the template (primer) is indicated beside the final time points. Other details are given in Materials and Methods.

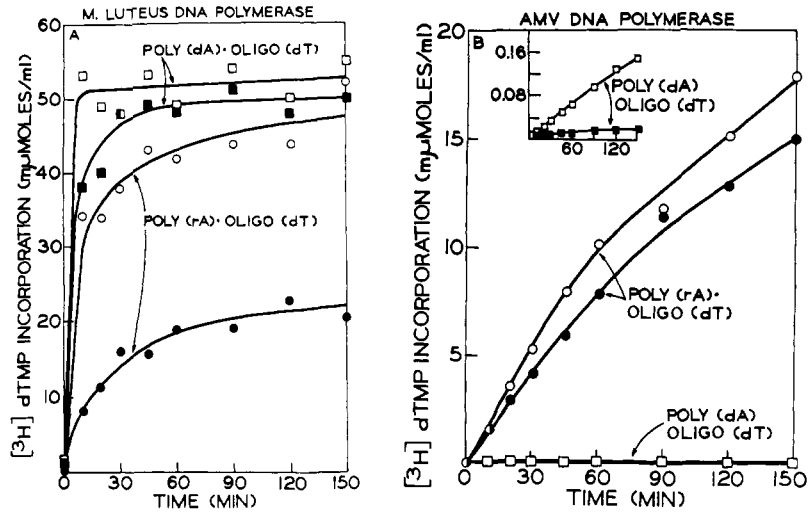

FIGURE 4: Kinetics of $(dT)_n$ synthesis with oligo(dT) as a primer and either $(dA)_n$ or $(rA)_n$ as template. For both parts A and B, reactions were performed with $(dA)_n$, 43 μM (□, ■) and $(rA)_n$, 44 μM (○, ●) at two levels of $(pT)_{13}$ which were 32 μM (open symbols) and 1.1 μM (filled symbols). Other conditions are given in Materials and Methods. Note the expanded scale of the insert in B.

(3) Double-stranded DNAs are more active than homologous double-stranded RNAs. For example, compare $(dG)_n \cdot (dC)_n$ to $(rG)_n \cdot (rC)_n$. Lee-Huang and Cavalieri (1964) previously showed that $(rG)_n \cdot (rC)_n$ was relatively ineffectual with the *E. coli* DNA polymerase.

$(dA\text{-}dT)_n \cdot (dA\text{-}dT)_n$ is the most effective template (primer) tested for both the *M. luteus* and the AMV polymerases. This is not true for the *E. coli* DNA polymerase (Figure 1) and is due, no doubt, to the relatively high nuclease activity (Deutscher and Kornberg, 1969) of the *E. coli* DNA polymerase. Both the *M. luteus* and the AMV DNA polymerase have quite low amounts of nucleolytic activity (Harwood *et al.*, 1970b; Kacian *et al.*, 1971). $(dA\text{-}dT)_n \cdot (dA\text{-}dT)_n$ is a better nuclease substrate than $(dA)_n \cdot (dT)_n$ (Burd and Wells, 1970).

(4) For the hybrid polymers, the poly(r-purine)·poly(d-pyrimidine) is more active than the poly(d-purine)·poly(r-pyrimidine) in virtually every case. $(rA)_n \cdot (dT)_n$ is more active than $(dA)_n \cdot (rU)_n$ and $(rG)_n \cdot (dC)_n$ is more active than $(dG)_n \cdot (rC)_n$ (Tables I and II). The poly(r-purine)·poly(d-pyrimidine) hybrids are essentially as active as the homologous DNA·DNA polymers. It should be noted that $(rA)_n \cdot (dT)_n$ is apparently considerably more active than $(dA)_n \cdot (dT)_n$ for the AMV polymerase (Table I). However, the molar incorporations of dAMP and dTMP are approximately equal when $(rA)_n \cdot (dT)_n$ is the template (primer). Thus, the rather low incorporation of dTMP with the DNA duplex is the anomalous finding rather than the relatively greater incorporation in the case of $(rA)_n \cdot (dT)_n$. (It should be noted at this point that we have observed highly irreproducible incorporations with the AMV DNA polymerase in the case of $(rA)_n \cdot (dT)_n$. The reaction either proceeds quite well (Table I) or not at all. This behavior is observed only with the AMV polymerase and only in the case of this polymer.)

(5) For the DNA·RNA hybrids, the DNA strand serves as a template at least as well, or better, compared to the RNA strand. Study of the rate of synthesis of both complementary strands with the three enzymes using all four homopolymer hybrids (Tables I and II) provides this result. This general conclusion with polymeric templates (primers) is not in accord with the generalization of Baltimore and Smoler (1971) with polymer·oligomer mixtures. However, we have repeated and extended some of their studies (see below) and it is apparent that both conclusions are valid and depend on the types of templates (primers) used.

(6) For the homopolymeric DNA·DNA duplexes, the poly(d-pyrimidine) strand is generally synthesized to a lesser extent than the poly(d-purine) strand. Whereas this behavior is especially true for the AMV DNA polymerase (Table II), it is also found for the *M. luteus* enzyme and for the *E. coli* DNA polymerase in the case of $(dG)_n \cdot (dC)_n$ (Radding *et al.*, 1962).

These studies imply that RNA may serve as a primer for DNA synthesis.

All experiments shown in Tables I and II were also performed with Nonidet P-40 disrupted virions of AMV (0.25% NP-40 in 0.02 M dithiothreitol) as well as a more highly purified fraction (fraction IV) (results not shown). The relative templating ability was the same with all three enzyme preparations. Hence, at least for these templates (primers), it is concluded that the purified polymerase has the same capabilities as the crude system; *i.e.*, no factors were apparently lost during the purification steps.

Reactions with Oligonucleotide Primers. A recent paper (Baltimore and Smoler, 1971) indicates that crude preparations of RNA tumor virus DNA polymerase(s) have unique template (primer) specificities when short-chain deoxyribooligonucleotides are tested in conjunction with their complementary polydeoxy- or polyribonucleotides. Since this result is not consistent with our findings using high molecular weight polymers, we performed similar experiments with purified enzymes, which confirm and extend Baltimore's observation.

Figure 4A shows the kinetics of the *M. luteus* DNA polymerase reactions with oligo-$d(pT)_{13}$ as the primer in conjunction with either $(dA)_n$ or $(rA)_n$ as the template. Both $(dA)_n \cdot$ oligo(dT) and $(rA)_n \cdot$ oligo(dT) are suitable templates (primers); previous studies have shown that such mixtures will form stable complexes (Byrd *et al.*, 1965; Cassani and Bollum, 1969). The polydeoxyribonucleotide template is somewhat more effective than the polyribo template. Since only dTTP is present, the extent of the reaction levels off at approximately the level of the polypurine template (Jovin and Kornberg, 1968; Harwood and Wells, 1970)

Contrastingly, Figure 4B shows that approximately 100 times more synthesis is observed with $(rA)_n \cdot$ oligo(dT) than

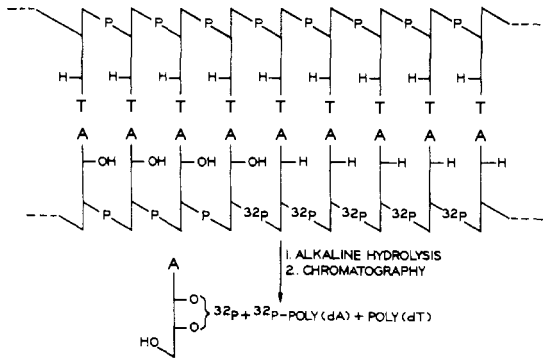

FIGURE 5: Outline of method used to demonstrate the joining of a newly synthesized DNA strand to an RNA primer in a DNA·RNA hybrid templated reaction. The figure illustrates the $(rA)_n \cdot (dT)_n$ templated (primed) reaction performed with the *M. luteus* DNA polymerase (see Figure 6). An analogous experiment was performed with the AMV DNA polymerase using $(rG)_n \cdot (dC)_n$ as a template (primer).

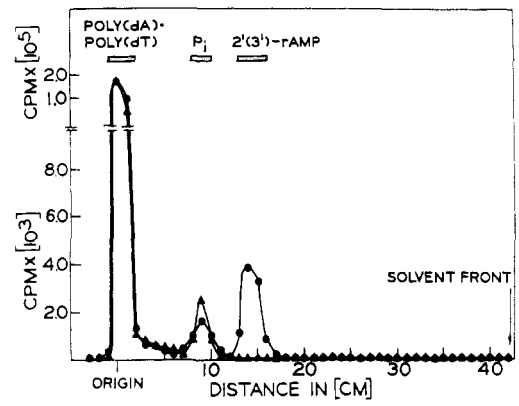

FIGURE 6: Radiochromatogram of degradation products after alkali treatment of products of the *M. luteus* DNA polymerase reaction. (▲) $(dA)_n \cdot (dT)_n$ as template (primer); (●) $(rA)_n \cdot (dT)_n$ as template (primer).

with $(dA)_n \cdot$oligo(dT) when the AMV DNA polymerase is tested. However, the insert to this figure demonstrates that the reaction with $(dA)_n \cdot$oligo(dT) is genuine but quite slow by comparison. The extent of synthesis with $(dA)_n \cdot$oligo(dT) is approximately the same as with $(dA)_n \cdot (dT)_n$ as the template (Table I). However, the extent of synthesis in 2 hr with $(rA)_n \cdot$oligo(dT) is four times greater than with $(rA)_n \cdot (dT)_n$ as template. As pointed out earlier, dTMP incorporation is rather low when $(dA)_n \cdot (dT)_n$ is the template (primer). No detectable reaction is found when $(dA)_n$, $(rA)_n$, or oligo(dT) were tested individually with either enzyme.

When dATP was added, in addition to dTTP as a substrate (each at 360 μM), in a $(rA)_n \cdot$oligo(dT) templated (primed) reaction with the AMV DNA polymerase, no detectable (<1% of dTMP incorporation) synthesis of $(dA)_n$ was observed (results not shown). Similarly, in a $(dA)_n \cdot$oligo(dT) templated reaction, dAMP was poorly incorporated (<10% of dTMP incorporation). Paper chromatography of the reaction mixture demonstrated that the dATP was not broken down during the incubation.

On the other hand, when $(rA)_n \cdot$oligo(dT) was tested with the *M. luteus* DNA polymerase in the presence of both dATP and dTTP, the dAMP was incorporated (85% of dTMP). Also when $(dA)_n \cdot$oligo(dT) was tested with this enzyme in the presence of both dNTP's, dAMP was incorporated 60% as well as dTMP. Hence, the tumor virus DNA polymerase differs from the bacterial polymerase in (1) its inability to utilize $(dA)_n \cdot$oligo(dT) as a template (primer) and (2) its inability to form $(dA)_n$ under the conditions specified. Hence, it may be possible to distinguish tumor virus polymerases from other polymerases with some template·primer mixtures. After submission of this work, a paper appeared containing similar findings (Goodman and Spiegelman, 1971).

DNA Product is Covalently Joined to RNA (or DNA) Primer. Previous studies with DNA polymerases have demonstrated that the newly synthesized DNA strand is covalently joined to the obligate deoxyribonucleotide primer (Bollum, 1962; Goulian, 1968; Harwood and Wells, 1970). The studies presented above with both polymer and oligonucleotide systems suggest that the newly synthesized DNA strand may also be joined to an RNA primer. If this is true, one radioactive 2'(3')-ribomonophosphate should be detectable, after alkaline hydrolysis of the product, for each new DNA strand when RNA serves as the primer (Figure 5). However, no radioactive monophosphate should be detected when DNA is the primer, instead of RNA.

Figure 6 shows the products after alkali treatment of the *M. luteus* DNA polymerase reaction product. In the control reaction in which $(dT)_n$ was the template and $(dA)_n$ was the primer in the presence of [α-^{32}P]dATP, the chromatogram shows two peaks; 97.9% of the isotope is in polymer product at the origin and 1.9% comigrates with P_i. No isotope could be detected in the AMP region (<0.2%).

In the $(rA)_n$ primed experiment, an additional peak was observed which comigrated with 2'(3')-rAMP and contained 3.2% of the total radioactivity. To further substantiate that the labeled product which comigrates with the 2'(3')-rAMP marker is not due to 5'-dAMP (or other compounds), a different solvent system was employed. In system A (Figure 6), 5'-dAMP has a lower mobility than 2'(3')-rAMP. On PEI-cellulose plates, the order of mobilities is reversed. Chromatography of the products in this thin-layer system showed a substantial amount of ^{32}P comigrating with 2'(3')-rAMP. However, no ^{32}P activity could be detected in the 5'-dAMP region. This result unequivocally shows that the $(rA)_n$ primer was covalently bonded to the newly synthesized $(dA)_n$ strand through a phosphodiester linkage.

Radioactive P_i was found in all analyses. To substantiate that this product was P_i, the substance was eluted from the paper and shown to be nonadsorbable to charcoal. Also, it had the same mobility as P_i on PEI-plates and in paper chromatography in solvent B. Its formation is presumably due to unspecific dephosphorylation after chain scission during the alkaline hydrolysis.

Similar studies were performed with the AMV DNA polymerase (Materials and Methods). $(dC)_n$ was the template in conjunction with either $(dG)_n$ or $(rG)_n$ as primer; [α-^{32}P]dGTP was the substrate in both cases. After the enzymatic reaction and isolation of the products, only polymeric product was detectable by paper chromatographic analyses. When $(dG)_n$ was the primer, essentially all (99.8%; 4.84 $\times$ 10^6 cpm) of the radioactivity remained at the origin after alkaline hydrolysis of the reaction products and subsequent paper chromatography. Some isotope (0.12%; 6150 cpm) was found at the position of rGMP in this control experiment and is due to the relatively high background due to trailing in the phosphate-containing system (solvent B) used for analysis. However, in the $(rG)_n$ primed experiment, greater than ten times as much

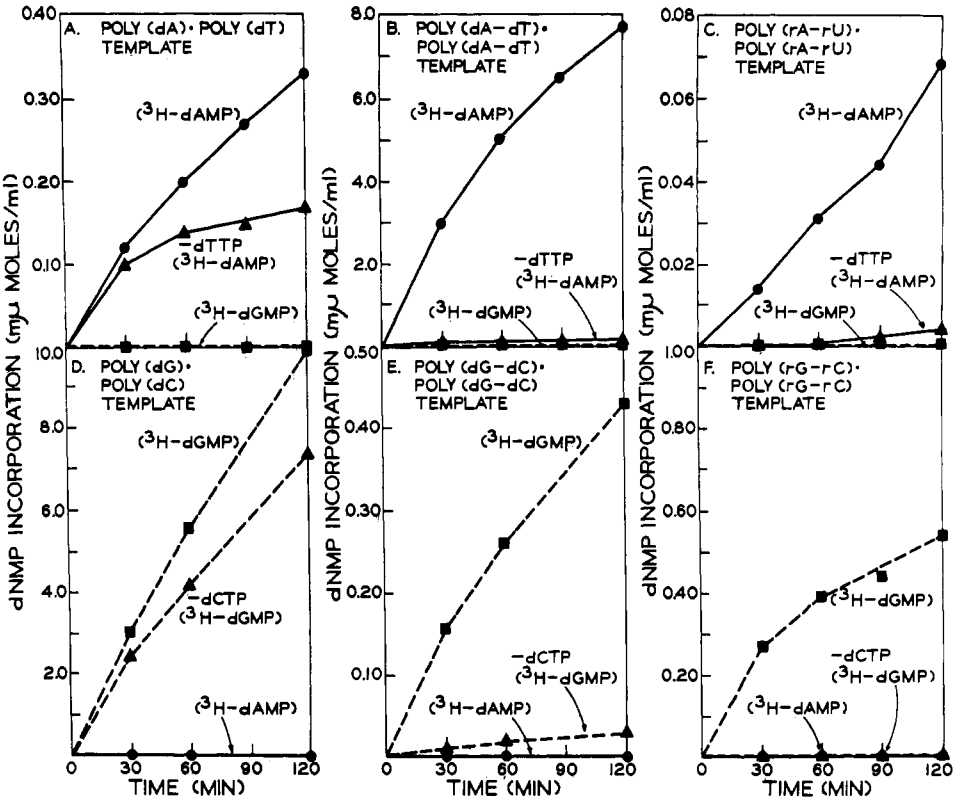

FIGURE 7: Faithfulness of AMV DNA polymerase as determined by dependence of dNMP incorporation on the presence of other substrates. The template (primer) for each set of reactions is indicated in each panel. Reactions contained all four dNTP's unless indicated otherwise (in which case one substrate was omitted). The ^{3}H-labeled nucleotide is indicated for each curve. (●—●) dAMP incorporation in presence of all four dNTP's; (▲—▲) dAMP incorporation in presence of dATP, dGTP, and dCTP; (●---●) dGMP incorporation in presence of all four dNTP's; (▲---▲) dGMP incorporation in presence of dATP, dTTP, and dGTP.

isotope was found at the position of 2′(3′)-rGMP (31,920 cpm; 1.4% of total radioactivity). That this product was 2′-(3′)-rGMP, and not another compound, was shown by chromatography in three systems. Hence the AMV DNA polymerase also catalyzes the covalent attachment of the DNA product to the RNA primer molecule. After submission of this work, a paper appeared indicating that a covalently linked DNA–RNA is formed in an AMV endogenous reaction (Verma *et al.*, 1971).

Although these studies demonstrate that the AMV DNA polymerase *can* covalently join a newly synthesized strand onto a primer strand, it may also be able to initiate the *de novo* synthesis of a new strand. It is possible that the enzyme demands a double-stranded region for initiation of DNA synthesis. Hence, experiments were performed with [γ-^{32}P]dGTP in a $(dG)_n \cdot (dC)_n$-templated reaction. A level of radioactivity (20×10^6 cpm/reaction) was used such that one initiation event per 2000 nucleotides incorporated into product was detectable. Under several different reaction conditions, it was not possible to detect the incorporation of [γ-^{32}P]dGTP into acid-insoluble DNA. The radioactive triphosphate was not degraded.

AMV DNA Polymerase Gives Faithful DNA Synthesis. To study the fidelity of replication, we tested the substrate incorporations with four DNAs and two RNAs; the possible incorporation of incorrect nucleotides (*i.e.*, not base paired to template) was examined (Figure 7). The defined nucleotide sequence of each of these polymers has been unequivocally demonstrated (Burd and Wells, 1970; Chamberlin *et al.*, 1963; Radding *et al.*, 1962; Wells *et al.*, 1970; DeClercq *et al.*, 1971). Due to the relatively low extent of DNA synthesis, this was deemed to be the most critical analysis to test the fidelity of replication. A previous study (Spiegelman *et al.*, 1970) indicated that the crude DNA polymerase (a detergent-disrupted tumor virus preparation) was faithful as judged by a nearest neighbor frequency study with only one labeled substrate. Hybridization studies also led to this conclusion but the level of sensitivity was very low in both types of assays.

Figure 7 shows that when a polymer with strictly alternating nucleotide sequence was the template (primer), nucleotide incorporation was dependent on the presence of both base-pair triphosphates that were complementary to the DNA. However, when homopolymer pairs were the templates (primers) (Figure 7A,D), the only requirement for incorporation was for the substrate to base pair with either DNA strand.

These studies establish that the AMV DNA polymerase faithfully replicates both DNA and RNA polymers of defined sequences.

It was previously shown that the *M. luteus* DNA polymerase is faithful when using DNA polymers as templates (primers) (Harwood *et al.*, 1970a). However, no information was available on the fidelity of this enzyme when ribopolymers were substituted as templates (primers). Thus, experiments similar to those described above were performed using $(rA\text{-}rU)_n \cdot (rA\text{-}rU)_n$ and $(rG\text{-}rC)_n \cdot (rG\text{-}rC)_n$ as templates (results not shown). In both cases, synthesis was dependent on the presence of both complementary dNTP's and a dNTP which was not complementary to the template was not incorporated. Thus, the *M. luteus* DNA polymerase makes faithful DNA copies of RNA polymers.

Discussion

Previous studies have been reported (Kornberg, 1969; Harwood *et al.*, 1970a; Spiegelman *et al.*, 1970; Baltimore and Smoler, 1971) on the effectiveness of some different templates with these DNA polymerases. However, due to recent inferences that the tumor virus associated DNA polymerases are unique in their ability to use RNA templates, a careful study under comparable conditions with a wide variety of templates (primers) was performed. On the basis of template preference studies with high molecular weight nucleic acids, the *E. coli* and the *M. luteus* DNA polymerases are at least as effective "reverse transcriptases" as the AMV DNA polymerase. Clearly, none of these three enzymes should be termed "RNA-dependent" polymerases but may be called "RNA directed."

However, the AMV DNA polymerase does have a unique template preference when oligo(dT) was tested in conjunction with $(rA)_n$. This behavior was reported previously (Baltimore and Smoler, 1971). $(rA)_n \cdot$ oligo(dT) is approximately 100-fold more active than $(dA)_n \cdot$ oligo(dT) when dTTP is the substrate. This dramatic difference is not observed for the *M. luteus* polymerase. The explanation for this behavior is uncertain at present.

The AMV DNA polymerase has other properties which are strikingly similar to the two bacterial DNA polymerases. (1) All three enzymes give faithful synthesis using a variety of templates with G + C contents varying from 0 to 100% (Kornberg, 1969; Harwood and Wells, 1970; R. C. Grant, M. Kodama and R. D. Wells, unpublished work). (2) A primer strand is necessary in addition to a template strand for all three enzymes and the newly synthesized DNA strand is covalently joined to the primer through a phosphodiester linkage. (3) It is apparently not possible for any of these three polymerases to effect *de novo* initiation of a new DNA strand in templated reactions as measured by retention of a triphosphate group at the 5′ end of the chain.

The finding that the DNA polymerases catalyze the covalent attachment of a new DNA strand to an RNA primer is of significance with regard to both initiation of cellular DNA synthesis and the possible involvement of tumor virus DNA polymerases in host cell transformation. These results suggest that the tumor virus enzyme may use as a primer the 3′-OH end of viral RNA which has looped back on itself. A similar mechanism was proposed (Goulian *et al.*, 1968) for single-stranded DNA-primed synthesis with the phage T_4 induced DNA polymerase. Alternatively, a separate RNA primer molecule may be complexed to the viral RNA template. Also, these findings raise the possibility that an RNA molecule may serve as a primer for cellular DNA synthesis; presumably, the RNA segment then would be cleaved from the polydeoxyribonucleotide strand.

Acknowledgment

We thank Dr. Joseph W. Beard, Department of Surgery, Duke University, for generously providing the avian myeloblastosis virus.

References

Baltimore, D. (1970), *Nature (London) 226*, 1209.
Baltimore, D., and Smoler, D. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 1507.
Bollum, F. J. (1962), *J. Biol. Chem. 237*, 1945.
Burd, J. F., and Wells, R. D. (1970), *J. Mol. Biol. 53*, 435.
Byrd, C., Ohtsuka, E., Moon, M. W., and Khorana, H. G. (1965), *Proc. Nat. Acad. Sci. U. S. 53*, 79.
Cassani, G. R., and Bollum, F. J. (1969), *Biochemistry 8*, 3928.
Chamberlin, M., Baldwin, R. L. and Berg, P. (1963), *J. Mol. Biol. 7*, 334.
DeClercq, E., Wells, R. D., Grant, R. C., and Merigan, T. C. (1971), *J. Mol. Biol. 56*, 83.
Deutscher, M. P., and Kornberg, A. (1969), *J. Biol. Chem. 244*, 3029.
Duesberg, P., Helm, K. v. d., and Canaani, E. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 747.
Fujinaga, K., Parsons, J. T., Beard, J. W., Beard, D., and Green, M. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 1432.
Goodman, N. C., and Spiegelman, S. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 2203.
Goulian, M. (1968), *Cold Spring Harbor Symp. Quant. Biol. 33*, 11.
Goulian, M., Lucas, Z. J., and Kornberg, A. (1968), *J. Biol. Chem. 243*, 627.
Harwood, S. J., Schendel, P. F., Miller, L. K., and Wells, R. D. (1970b), *Proc. Nat. Acad. Sci. U. S. 66*, 595.
Harwood, S. J., Schendel, P. F., and Wells, R. D. (1970a), *J. Biol. Chem. 245*, 5614.
Harwood, S. J., and Wells, R. D. (1970), *J. Biol. Chem. 245*, 5625.
Hatanaka, M., Huebner, R. J., and Gilden, R. V. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 143.
Inman, R. B. (1964), *J. Mol. Biol. 9*, 624.
Jovin, T. M., Englund, P. T., and Bertsch, L. L. (1969), *J. Biol. Chem. 244*, 2996.
Jovin, T. M., and Kornberg, A. (1968), *J. Biol. Chem. 243*, 250.
Kacian, D. L., Watson, K. F., Burny, A., and Spiegelman, S. (1971), *Biochim. Biophys. Acta 246*, 365.
Khorana, H. G., and Connors, W. J. (1966), *Biochem. Prep. 11*, 113.
Kornberg, A. (1969), *Science 163*, 1410.
Lee-Huang, S., and Cavalieri, L. F. (1963), *Proc. Nat. Acad. Sci. U. S. 50*, 1116.
Lee-Huang, S., and Cavalieri, L. F. (1964), *Proc. Nat. Acad. Sci. U. S. 51*, 1022.
Miller, L. K., and Wells, R. D. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 2298.
Mizutani, S., Boettinger, D., and Temin, H. M. (1970), *Nature (London) 228*, 424.
Mizutani, S., and Temin, H. M. (1971), *J. Virology 8*, 409.
Mizutani, S., Temin, H. M., Kodama, M., and Wells, R. D. (1971), *Nature New Biol. 230*, 232.
Radding, C. M., Josse, J., and Kornberg, A. (1962), *J. Biol. Chem. 237*, 2869.
Riley, M., Maling, B., and Chamberlin, M. J. (1966), *J. Mol. Biol. 20*, 359.
Riman, J., and Beaudreau, G. S. (1970), *Nature (London) 228*, 427.
Roy, P., and Bishop, D. H. L. (1971), *Biochim. Biophys. Acta 235*, 191.
Scolnick, E. M., Aaronson, S. A., Todaro, G. J., and Parks, W. P. (1971), *Nature (London) 229*, 318.
Scolnick, E., Rands, E., Aaronson, S. A., and Todaro, G. J. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 1789.
Spiegelman, S., Burny, A., Das, M. R., Keydar, J., Schlom, J., Travnicek, M., and Watson, K. (1970), *Nature (London) 227*, 563.
Studier, F. W. (1965), *J. Mol. Biol. 11*, 373.
Temin, H. M., and Mizutani, S. (1970), *Nature (London) 226*, 1211.

Verma, I. M., Meuth, N. L., Bromfeld, E., Manly, K. F., and Baltimore, D. (1971), *Nature New Biol. 233*, 131.
Wells, R. D., and Blair, J. E. (1967), *J. Mol. Biol. 27*, 273.
Wells, R. D., Jacob, T. M., Narang, S. A., and Khorana, H. G. (1967), *J. Mol. Biol. 27*, 237.
Wells, R. D., and Larson, J. E. (1970), *J. Mol. Biol. 49*, 319.
Wells, R. D., Larson, J. E., Grant, R. C., Shortle, B. E., and Cantor, C. R. (1970), *J. Mol. Biol. 54*, 465.

Transfer Ribonucleic Acid Methylases of Nucleoli Isolated from a Rat Tumor*

Ming C. Liau, Cathleen M. O'Rourke, and Robert B. Hurlbert†

ABSTRACT: When nucleoli were isolated from Novikoff ascites tumor cells of the rat and incubated with L-[*methyl*-^{3}H]S-adenosylmethionine in a suitable medium, incorporation of the methyl groups into the ribose of preribosomal RNA was observed. When *Escherichia coli* tRNA was added to the medium, this exogenous tRNA was methylated in addition, but the label was located on the purine and pyrimidine bases. The enzymes responsible for methylation of the bases of heterologous tRNA were extracted with 0.5 M NH_4Cl medium from the nucleoli and compared with pH 5-precipitated tRNA methylases derived mainly from cytoplasmic or soluble portions of the cells. The nucleolar enzymes represented about 5% of the total cellular tRNA methylase activity extractable from the cells. The activities of the nucleolar and "cell-soluble, pH 5" preparations were similar in response to pH, ionic strength, and ATP concentration, and differed somewhat in response to NaF and $MgCl_2$ concentrations. The major products with both preparations were 1-methyladenine, 7-methylguanine, 1-methylguanine, N^2-methylguanine, N^2-dimethylguanine, 5-methylcytosine, thymine, and 5-hydroxymethyluracil. The proportion of the methylated bases differed considerably; for example, with the nucleolar preparation 69% of the label was found in methylcytosine and 15% in methylguanines, while with the cell-soluble, pH 5 enzyme, 13% was in methylcytosine and 60% was in the guanine derivatives. When cells were labeled *in vivo* with [*methyl*-^{3}H]-methionine, the patterns of labeling of endogenous 4–6S RNAs had the same trend; for example, 50% of the nucleolar sRNA methyl groups were in cytosine derivatives and 53% of the cytoplasmic sRNA methyl groups were in guanine derivatives. The nucleolar tRNA methylation enzymes were judged to be true components of the nucleoli and to form a distinct subgroup of the total cellular methylation enzymes. It is suggested that they function as part of a nucleolar protein-synthesizing system.

The presence of an enzyme system for the methylation of endogenous precursor rRNA in isolated nucleoli of rat liver has been described by Culp and Brown (1970) and in isolated nucleoli of Novikoff ascites tumor cells by Liau *et al.* (1970). In contrast to the liver nucleoli, nucleoli isolated from Novikoff ascites hepatoma cells contained an additional group of enzymes which transferred methyl groups from *S*-adenosylmethionine into the nucleotide bases of an exogenous heterologous tRNA, *i.e.*, *Escherichia coli* tRNA. Birnstiel *et al.* (1963) have also demonstrated the ability of heterologous tRNA to stimulate the methylation of RNA by nucleolar preparations from pea seedlings.

The study of tRNA methylases has been a subject of interest because of the essential role that tRNAs and their modified subspecies play in protein synthesis and its regulation (Craddock, 1970), in differentiation (Baliga *et al.* 1965; Pillinger and Borek, 1969; Turkington, 1969), and possibly in malignant transformation (Borek, 1969). These enzymes are strictly specific for the individual bases (Srinivasan and Borek, 1963; Hurwitz *et al.*, 1964) and the pattern and extent of methyl distribution in the tRNA population is specific for species. The specificity appears also to be dependent on the sequence of bases containing the base to be methylated (Baguley and Staehelin, 1969; Baguley *et al.*, 1970; Kuchino and Nishimura, 1970).

Whereas tRNA methylase activity appears predominantly in the soluble fraction of mammalian cells (Burdon *et al.*, 1967; Muramatsu and Fujisawa, 1968; Culp and Brown, 1968), evidence is accumulating that nuclei or nuclear components, particularly nucleoli, show some enzymic activity (Birnstiel *et al.*, 1963; Sirlin *et al.*, 1966; Burdon *et al.*, 1967; Culp and Brown, 1968). Information on these enzymes localized in the nucleus is strikingly lacking; the bulk of study on tRNA methylases of mammalian cells has been conducted on enzymes obtained from the soluble fraction (Rodeh *et al.*, 1967; Kaye and LeBoy, 1968; Gantt and Evans, 1969; Moore and Smith, 1969; Kerr, 1970). The present study demonstrates that isolated nucleoli of Novikoff ascites hepatoma contain a small but significant amount of tRNA methylases, which are easily extractable and have characteristics generally similar to those of enzymes present in the soluble fraction. Evidence is presented to show that the nucleolar enzyme preparation does however methylate the *E. coli* tRNA bases in distinctly dif-

* From the Department of Biochemistry, The University of Texas at Houston, M. D. Anderson Hospital and Tumor Institute, Texas Medical Center, Houston, Texas 77025. *Received September 8, 1971.* This work was supported by U. S. Public Health Grant CA-10244 and by American Cancer Society Grant E-609.

† Author to whom correspondence should be addressed.