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# *In vitro* discrimination of replicases acting on carcinogen-modified polynucleotide templates

(*Escherichia coli* DNA polymerase I/cucumber RNA-dependent RNA polymerase/chloroacetaldehyde/dimethyl sulfate)

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**ABSTRACT** **Three different poly(dC)s with modifications that block the N-3 of deoxycytidine were used as templates for polymer synthesis by *Escherichia coli* DNA polymerase I (EC 2.7.7.7). In contrast to previously reported results with transcriptases, the hydrated form of 3,$N^4$-ethenodeoxycytidine (εdC·$H_2O$) did not mispair. Both 3,$N^4$-ethenodeoxycytidine (εdC) and 3-methyldeoxycytidine ($m^3$dC) led to dTMP misincorporation: 1/20 εdC and 1/80 $m^3$dC. No other misincorporations appeared to be significant in amount. Thus, both qualitatively and quantitatively, replication errors resulting from carcinogen-modified bases are less frequent than errors in transcription of the same deoxypolynucleotides. Replication of comparable ribopolynucleotide templates by cucumber RNA-dependent RNA polymerase (EC 2.7.7.48) was strongly inhibited by εrC·$H_2O$ and εrC, so that the fidelity of this enzyme could not be assessed. However, both poly(dC) and poly(rC) containing dU or rU led to incorporation of rA. The presence of even small amounts of purines in poly(rC) greatly depressed synthesis, but the complementary base was incorporated. The finding that an RNA replicase can utilize a deoxypolynucleotide template is a further indication that, at least *in vitro*, the specificity of the relationship of enzymes and their natural templates is not absolute.**

The potential mutagenic effects of carcinogen modification of nucleic acids have been studied in model systems, generally by assessing miscoding with synthetic polynucleotides as templates. Most of this work has been done with DNA-dependent RNA polymerases, relying on the fact that these enzymes have the ability to utilize ribopolynucleotides in the presence of $Mn^{2+}$ (for reviews, see refs. 1 and 2). More recently we reported that similar results on the miscoding potential of modified cytosines can also be obtained with the normal cation, $Mg^{2+}$, with both ribo- and deoxyribopolynucleotide templates (3).

These studies using transcriptases have now been extended to the use of replicases, enzymes that normally duplicate polynucleotides, making DNA on a DNA template or RNA on a RNA template.

*Escherichia coli* DNA polymerase I (pol I; EC 2.7.7.7) copies unmodified templates with high fidelity (for review, see ref. 4) but has been shown to misincorporate nucleotides on depurinated (5, 6) and depyrimidinated (7) templates. In the case of templates treated with carcinogens, there are no reports dealing with errors resulting from a single type of modified base because the templates used in such studies are likely to contain multiple sites of modification. Ideally, appropriate copolymers should be synthesized, as has been done for ribopolynucleotides, but there are technical problems, particularly with unstable derivatives, and few such defined polymers have been made for the study of miscoding (8–10). On the other hand, there are three potentially mispairing modifications on the N-3 of deoxycytidine that, under specific conditions, can be shown by HPLC analysis to be individually produced in poly(dC). These are the hydrated form of 3,$N^4$-ethenodeoxycytidine (εdC·$H_2O$), 3,$N^4$-ethenodeoxycytidine (εdC), and 3-methyldeoxycytidine ($m^3$dC). This paper deals with the effect of these modifications on the DNA polymerase I-directed synthesis of polynucleotides.

The second type of replicase, RNA-dependent RNA polymerase (EC 2.7.7.48), has only recently been purified from cucumber (11), and its ability to use various templates is compared with that of the DNA replicase.

## MATERIALS AND METHODS

**Preparation of Modified Polynucleotides.** Poly(dC), ≈3 *A* at $\lambda_{max}$ in 50 μl of 0.1 M pH 7.5 sodium cacodylate buffer, was allowed to react with 5 μl of 1:10 dilution of a 45% (vol/vol) aqueous solution of chloroacetaldehyde at 37°C for 1–15 min, depending on the extent of modification desired. Polymer isolation and analysis of the products was according to Kuśmierek and Singer (12). The chloroacetaldehyde-modified poly(dC) samples used were allowed to react 1, 3, and 15 min to obtain 1%, 2.6%, and 6.3% modification. Without further treatment the modification was found to be >95% in the form of hydrated εdC (12). Portions of the modified polymers were heated at 80°C in 0.01 M pH 7.25 Tris·HCl buffer in order to convert the hydrated εdC partially (30 min) or completely (60 min) to εdC. Chloroacetaldehyde-modified poly(rC) samples were the same as those used by Kuśmierek and Singer in a related study (3).

Dimethyl sulfate was used to methylate poly(dC). Five *A* at $\lambda_{max}$ of poly(dC) in 0.1 ml of 0.1 M pH 7.2 cacodylate buffer was allowed to react at 20°C for 3 hr with 2 μl or 5 μl of a 1:10 dilution of dimethyl sulfate in 90% (vol/vol) methanol. Repeated precipitation with ethanol was used to free the polymer from reagent. Digestion to nucleosides followed by HPLC (13) showed that the only detectable product was $m^3$dC (5%, 9%). rU or dU was introduced into poly(rC) or poly(dC) by nitrous acid deamination (14).

**Complementary Polynucleotide Synthesis and Nearest-Neighbor Analysis of the Products.** Purified *E. coli* DNA polymerase I, kindly supplied by L. A. Loeb, was used under the following conditions. In order to obtain a 10:1 template-to-primer ratio, which was experimentally determined to be most favorable, 0.15 $A_{260}$ unit of poly(dC) was mixed with 0.05 $A_{260}$ unit

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Abbreviations: εdC and εrC, 3,$N^4$-ethenodeoxycytidine and 3,$N^4$-ethenocytidine; εdC·$H_2O$ and εrC·$H_2O$, hydrated forms of the nucleosides; $m^3$dC, 3-methyldeoxycytidine. C, U, T, A, and G are used when only the base is of importance, regardless of the nature of the sugar and the presence of phosphate groups.

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of $(dG)_{15-18}$. The final volume of 0.625 ml of 0.1 M pH 7.8 Tris·HCl/2 mM $MgSO_4$/0.05 mM of each of the four dNTPs, contained 0.25 mCi (1 Ci = $3.7 \times 10^{10}$ Bq) of [$\alpha$-$^{32}$P]dGTP. After 5-min of incubation at 37°C, 5 units of polymerase was added and synthesis was allowed to proceed for 30 min at 37°C. Denatured calf thymus DNA (0.3 mg) was then added and the mixture was precipitated and washed according to Kuśmierek and Singer (3). The final precipitate was dissolved in 0.1 ml of 0.025 M NaOH. After thorough mixing, the pellet was kept at 37°C until completely in solution. The pH was adjusted to 7.5 by the addition of 0.06 ml of 1 M pH 6.8 Tris·HCl buffer followed by 0.02 ml of 0.2 M $CaCl_2$ and the polymers were digested at 37°C with a total of 0.3 unit of phosphodiesterase II (spleen exonuclease) and 450 units of micrococcal nuclease, added in three aliquots over an 8-hr period. In contrast to single-stranded DNA, digestion was not complete. Additional enzyme treatment did not lead to further digestion. There was no apparent correlation between extent of hydrolysis of polymers and their composition. The resulting 3′-deoxynucleotides were separated by electrophoresis in pH 3.5 pyridine acetate buffer (13). Each nucleotide was repurified by chromatography as follows. The UV-absorbing areas were cut out, sewn on Whatman 3MM paper, and subjected to descending chromatography in 2-propanol/1% ammonium sulfate, 2:1 (vol/vol). The $R_f$s of the 3′-deoxynucleotides (dTMP > dCMP, dAMP > dGMP) were in a different order than the electrophoretic mobilities (dTMP > dGMP > dAMP > dCMP), which facilitated separation of the major labeled nucleotide, dGMP, from the much smaller amounts of radioactivity in other nucleotides.

Cucumber RNA-dependent RNA polymerase, kindly supplied by Y. Takanami, was used as follows. The optimal conditions were 8 mM $MgSO_4$, 40 mM Tris·HCl at pH 8.0, 20 mM ammonium sulfate, 2 mM KCl, 1 mM dithiothreitol, and each ribonucleoside triphosphate at 0.4 mM. The 0.625-ml incubation mixture contained 0.15 $A_{260}$ unit of polymer and 0.025 mCi of [$\alpha$-$^{32}$P]GTP. Synthesis was allowed to proceed 60 min at 30°C. Nearest-neighbor analysis was according to Kröger and Singer (13).

## RESULTS

Nearest-neighbor analysis was used throughout because this method simultaneously yields absolute and relative data on the incorporation of all four nucleosides.

In Tables 1 and 2, the extent of synthesis, measured by complementary dGMP incorporation, is shown to be reduced by any treatment of the original homopolymer. However, the least effective template tested for DNA polymerase I [poly(dC, 9% $m^3$dC)] is still transcribed about 20% as well as poly(dC), whereas RNA-dependent RNA polymerase synthesis is sharply reduced by even small amounts of purines, and synthesis is almost completely inhibited by 9% $\varepsilon$rC.

Regardless of the extent of synthesis, both replicases copy normal modified bases, indicating that low overall synthesis probably reflects a decrease in rate. The RNA replicase can also utilize poly(dC) and poly(dC, 2.3% dU) (Table 1) as templates, though with less efficiency than the ribopolynucleotides.

The fidelity of synthesis using poly(dC) or poly(rC) templates is quite high with both enzymes, but the incorporation of A is always increased above that of C or U/T. This probably reflects the deamination occurring during storage or usage of C polymers. In Table 2 it can be seen that heating of poly(dC) increases the dAMP incorporation. For an unexplained reason, as the amount of etheno derivative increases, the heat-induced deamination decreases.

The only nucleotide incorporated in increasing amounts as the extent of modification increases is dTMP. This incorporation is a function of the amount of $\varepsilon$dC or $m^3$dC, but it is not observed to occur as a consequence of the presence of hydrated $\varepsilon$dC (Table 2). Fig. 1 shows the data after the background radioactivity found in the dTMP area with poly(dC) as template has been subtracted. The heating of poly(dC) containing hydrated $\varepsilon$dC leads to its gradual loss of water, resulting in $\varepsilon$dC, which leads to dTMP misincorporation. Approximately 1 dTMP is incorporated for 20 $\varepsilon$dC residues.

The other modification of the N-3 of dC also results in dTMP misincorporation but less frequently, about 1/80 $m^3$dC. Deamination, as expected, increases the dAMP incorporation only.

## DISCUSSION

The hydrated form of $\varepsilon$C has been shown to be an intermediate in the formation of $\varepsilon$C. Only recently it has been reported that under physiological conditions the hydrate is quite stable in polynucleotides, with a $t_{1/2}$ of about 13 hr at 37°C (12). Therefore, in cells exposed to vinyl chloride, in which chloroacetaldehyde is a metabolite, the hydrate would be expected to be present. Because this compound lacks the fluorescence that is characteristic of $\varepsilon$C, it can easily be overlooked.

Table 1. Nearest-neighbor analysis of products synthesized by replicases using poly(rC) or poly(dC) templates

| | *E. coli* DNA polymerase I | | | | Cucumber RNA-dependent RNA polymerase | | | |
|---|---|---|---|---|---|---|---|---|
| Polymer | dGMP, cpm × $10^{-4}$ | (dCMP/dGMP) × $10^2$ | (dAMP*/dGMP) × $10^2$ | (dTMP/dGMP) × $10^2$ | GMP, cpm × $10^{-3}$ | (CMP/GMP) × $10^2$ | (AMP*/GMP) × $10^2$ | (UMP/GMP) × $10^2$ |
| Poly(dC) | 954 | 0.07 | 0.5 | 0.18 | 767 | 0.08 | 0.3 | 0.04 |
| Poly(rC) | | | | | 1,523 | 0.09 | 0.2 | 0.04 |
| Poly(dC, 2.3% dU) | 424 | 0.13 | **4.1** | 0.14 | 436 | 0.09 | **2.7** | ND |
| Poly(rC, 4% rU) | | | | | 708 | 0.13 | **5.8** | 0.1 |
| Poly(rC, 6% rG) | | | | | 127 | **6.9** | 0.3 | ND |
| Poly(rC, 12% rA) | | | | | 78 | 0.28 | 0.5 | **4.9** |
| Poly(dC, 6.3% $\varepsilon$dC·$H_2O$) | 257 | 0.04 | 0.3 | 0.16 | | | | |
| Poly(dC, 6.3% $\varepsilon$dC) | 302 | 0.04 | 0.4 | **0.40**† | | | | |
| Poly(dC, 8% $\varepsilon$dC·$H_2O$) | | | | | 37 | | | |
| Poly(dC, 8% $\varepsilon$dC) | | | | | 21 | | | |
| Poly(rC, 9% $\varepsilon$rC·$H_2O$) | | | | | 25 | | | |
| Poly(rC, 9% $\varepsilon$rC) | | | | | 25 | | | |

Significant incorporation is in boldface. ND, not determined.

* dAMP and AMP incorporation, even in unmodified polymers, is higher than cytosine or uracil/thymine nucleotide incorporation, presumably as a result of low levels of background deamination (0.2–0.3%).

† The significance of this value is based on data from the series given in Table 2.

Table 2. Nearest-neighbor analysis of products synthesized by DNA polymerase I using poly(dC) modified by chloroacetaldehyde or dimethyl sulfate

| Polymer | Treatment* | dGMP, cpm × $10^{-4}$ | (dTMP/dGMP) × $10^2$ Observed | (dTMP/dGMP) × $10^2$ Corrected‡ | (dCMP/dGMP) × $10^2$ | (dAMP†/dGMP) × $10^2$ |
|---|---|---|---|---|---|---|
| Poly(dC) | None | 954 | 0.18 | | 0.08 | 0.5 |
| | Heated 30 min | 650 | 0.14 | | 0.08 | 1.0 |
| | Heated 60 min | 753 | 0.12 | | 0.07 | 1.2 |
| Poly(dC) | None | 409 | 0.17 | — | 0.07 | 0.6 |
| 1% chloroacetaldehyde | Heated 30 min | 428 | 0.17 | — | 0.06 | 0.5 |
| modified§ | Heated 60 min | 585 | 0.20 | 0.08 | 0.13 | 1.1 |
| Poly(dC) | None | 531 | 0.17 | — | 0.03 | 0.3 |
| 2.6% chloroacetaldehyde | Heated 30 min | 622 | 0.24 | 0.1 | 0.05 | 0.3 |
| modified§ | Heated 60 min | 517 | 0.23 | 0.11 | 0.04 | 0.3 |
| Poly(dC) | None | 257 | 0.16 | — | 0.04 | 0.3 |
| 6.3% chloroacetaldehyde | Heated 30 min | 180 | 0.30 | 0.16 | 0.07 | 0.3 |
| modified§ | Heated 60 min | 299 | 0.40 | 0.28 | 0.04 | 0.4 |
| Poly(dC, 5% $m^3dC$) | None | 200 | 0.23 | 0.05 | 0.06 | 0.3 |
| Poly(dC, 9% $m^3dC$) | None | 176 | 0.33 | 0.15 | 0.06 | 0.3 |

* Polymer was heated at 80°C in pH 7.25 0.01 M Tris·HCl buffer for the indicated times.
† The comparatively high and erratic dAMP values are attributed to the dU content resulting from deamination during storage and treatment.
‡ The value obtained with poly(dC) treated in parallel is subtracted.
§ Percent chloroacetaldehyde modification refers to the total amount of modified dC residues. Heating of chloroacetaldehyde-treated polymers converts the hydrated intermediate ($\varepsilon dC \cdot H_2O$) to εdC. Partial conversion occurs in 30 min; conversion is complete in 60 min.

Previous studies of errors attributed to εdC or $1,N^6$-ethenodeoxyadenosine (εdA) have utilized chloroacetaldehyde-treated poly(dA), poly(dC), poly(dA-dT), or poly(dC-dG) which were not analyzed for the amount of hydrate or, in the case of chloroacetaldehyde-treated poly(dC-dG), for modified dG. Using homopolymers, Barbin *et al.* (15) found that the chloroacetaldehyde-treated poly(dC) led to dTMP misincorporation, which agrees with our results, if one assumes that the reaction conditions used (20 hr, 37°C) dehydrated the intermediate. However, Hall *et al.* (16) used chloroacetaldehyde-treated poly(dC-dG) as template with DNA polymerase I and found both dAMP and dTMP misincorporated, to the extent of 1 in 30 and 80 εdC residues, respectively. It is likely that the template used by Hall *et al.* contained a significant amount of hydrated εdC, which would partially account for their lower misincorporation of dTMP. The recently described product of DNA reaction with chloroacetaldehyde, $3,N^2$-ethenodeoxyguanosine, is postulated to mispair with dT (17). Thus the report by Hall *et al.* (16) that by nearest-neighbor analysis dTMP was found equally opposite dC and dG is additional evidence that their polymer contained little εdC, but rather primarily hydrated εdC which we now find not to miscode. dAMP misincorporation was not detected in the present study.

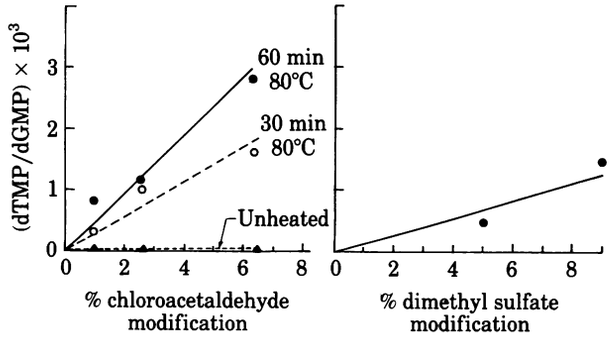


FIG. 1. Misincorporation of dTMP by *E. coli* DNA polymerase I using as templates poly(dC) modified by chloroacetaldehyde (*Left*) or dimethyl sulfate (*Right*). The modification in unheated chloroacetaldehyde sample is >95% hydrated εdC. Heating at 80°C for 60 min in 0.01 M pH 7.2 Tris·HCl quantitatively converts the hydrated form to εdC. The only modified base found by HPLC in dimethyl sulfate-treated poly(dC) is $m^3dC$.

It should be noted that in previous studies of transcription, a copolymer of rC and εrC resulted in misincorporation of UMP > AMP ≫ CMP (18). The total misincorporation with DNA-dependent RNA polymerases in the presence of $Mg^{2+}$ was similar to the amount of εrC, thus facilitating detection of errors occurring rarely. The transcriptases also led to misincorporation of AMP > UMP ≫ CMP with templates containing hydrated εdC (18), in sharp contrast to the lack of detectable misincorporation now observed with DNA polymerase I for deoxypolynucleotide synthesis. This latter clear qualitative difference in fidelity between transcriptases and DNA polymerase I is an indication that these enzymes play a key role in selection of the proper nucleoside triphosphate.

A surprising finding was that RNA-dependent RNA polymerase could also use as templates poly(dC) and copolymers containing unmodified bases. This type of enzyme was previously found to utilize single-stranded DNA to only a very low extent and double-stranded DNA not at all (19). Loeb *et al.* (20) had noted that DNA-dependent DNA polymerase also utilized RNA templates, although with lower efficiency. Similarly, DNA-dependent RNA polymerases utilize both ribo- and deoxyribopolymers as templates, although only poly(rC) is active as template in the presence of $Mg^{2+}$, the normal cation (3). It thus appears that the specificity of polymerizing enzymes for templates is not absolute.

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