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Articles

Template-Primer Activity of 5-(Hydroxymethyl)uracil-Containing DNA for Prokaryotic and Eukaryotic DNA and RNA Polymerases†

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Received February 15, 1989; Revised Manuscript Received June 16, 1989

ABSTRACT: We have utilized *Bacillus subtilis* phage SPO-1 DNA as a model of irradiated DNA. In this phage, all thymine (Thy) residues are replaced by 5-(hydroxymethyl)uracil (5HmUra), which is a known irradiation-induced derivative of DNA Thy. SPO-1 phage is naturally devoid of other such irradiation-induced DNA lesions. DNase I activated SPO-1 phage DNA served as well as, or even better than, the control DNAs (*Bacillus subtilis* DNA and calf thymus DNA) as a template-primer for *Escherichia coli*, *Micrococcus luteus*, and human HL-60 cell DNA polymerases. Futhermore, the template activity of SPO-1 phage DNA was also superior when transcription with *E. coli* RNA polymerase was investigated. The results reported here indicated that the replacement of Thy by 5HmUra is not deleterious to template and primer functions during DNA or RNA synthesis.

Three interesting situations are known when DNA Thy may partially or fully be replaced by 5HmUra: (i) 5HmUra is the natural constituent of DNA in certain *Bacillus subtilis* phages and dinoflagellates (Kallen et al., 1962; Okubo et al., 1964). (ii) 5HmUra is a known transmutation product in DNA of tritiated Thy (Teebor et al., 1984). It can also be induced by ionizing irradiation or other oxidative attack (Lewis et al., 1978; Frenkel et al., 1985; Frenkel & Chrzan, 1987; Teebor, et al., 1988). (iii) Finally, 5HmUra can be introduced into DNA from exogenous 5HmdUrd during semiconservative DNA replication (Matthes et al., 1979; Kahilainen et al., 1985; Kaufman, 1986; Boorstein et al., 1987; Vilpo & Vilpo, 1988).

We have investigated the biochemical consequences which might result if DNA Thy is replaced by 5HmUra. The experiments described in this report focus on the template-primer activity of 5HmUra-containing DNA. We have chosen *Bacillus subtilis* phage SPO-1 DNA for the experimental model, since all DNA Thy residues in this species are replaced naturally by 5HmUra (Kallen et al., 1962; Okubo et al., 1964). The results reported here demonstrate that the presence of 5HmUra does not compromise the template-primer functions of DNA; SPO-1 phage DNA is a good template-primer for various DNA and RNA polymerases when compared to the host (*Bacillus subtilis*) or calf thymus DNA.

† This work was supported by grants from the Finnish Foundation for Cancer Research and the Academy of Finland.
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Experimental Procedures

Materials

Nonradioactive triphosphates were purchased from Calbiochem-Behring or from Sigma. [8-^{3}H]dATP (13.1 Ci/mmol) and [5-^{3}H]UTP (11.0 Ci/mmol) were from Amersham. *Escherichia coli* DNA polymerase I (Kornberg polymerase, endonuclease free; EC 2.7.7.7), *E. coli* RNA polymerase (EC 2.7.7.6), *Hin*dIII-digested λ phage DNA (molecular weight markers), and aphidicolin were purchased from Boehringer Mannheim. Sigma was the supplier of *Micrococcus luteus* DNA polymerase (EC 2.7.7.7), pancreatic DNase I (EC 3.1.21.1), and highly polymerized calf thymus DNA.

Methods

Template-Primers. Bacillus subtilis and its phage SPO-1 (catalog no. 27370-B1) were obtained from the American Type Culture Collection (Rockville, MD). The phage was cultured

0006-2960/89/0428-8274$01.50/0

and DNA isolated as described previously (Kallen et al., 1962). ^{32}P postlabeling analysis (Wilson et al., 1986) and HPLC (Vilpo et al., 1987) did not reveal any thymidine in the SPO-1 DNA preparations, all of it being replaced by 5HmdUrd. *Bacillus subtilis* DNA was isolated by traditional methods (Clark & Switzer, 1977). Activated template-primers were prepared by exposing the DNAs to different concentrations of pancreatic DNase for 15 min at 37 °C as described by Aposhian and Kornberg (1962). The extent of hydrolysis of DNA was examined by gel electrophoresis on 0.8% neutral (for double-strand breaks) and alkaline (for nicks or single-strand breaks) agarose gels as described in detail by Maniatis et al. (1982).

HL-60 Cell Extract. The human promyelocytic leukemia cell line (HL-60) was a generous gift from Professor Leif Andersson (Department of Pathology, University of Helsinki). The cells were grown in RPMI 1640 culture medium containing glutamine (2 mM), penicillin (100 units/mL), streptomycin (100 μg/mL), and fetal calf serum (10% v/v). The other conditions were a humidified atmosphere containing 5% CO_2 at 37 °C. The whole cell extract was prepared from exponentially growing cells according to the procedure of Manley et al. (1983), who originally developed the system for assessment of eukaryotic cell transcription. Protein concentrations were determined as described by Lowry et al. (1951).

DNA Polymerase Reaction. The reaction mixture for template-primer-dependent DNA synthesis (total volume 50 μL each) contained 50 mM Tris-HCl (pH 7.5)/10 mM $MgCl_2$/2 mM dithiothreitol/10 μM each of dTTP, dCTP, dGTP, and [^{3}H]dATP (0.1 μCi/assay) as well as the indicated amounts of DNA polymerase and template-primer DNA. The reaction was stopped and DNA precipitated, along with 100 μg of carrier DNA, with 0.2 N perchloric acid. The precipitate was washed twice with 0.2 N perchloric acid and dissolved in 10 μL of 1 N NaOH and 500 μL of water, and the radioactivity was determined by liquid scintillation counting, with a counting efficiency of approximately 25%.

RNA Polymerase Reaction. The reaction mixture contained (total volume 50 μL of each) 40 mM Tris-HCl (pH 7.9)/10 mM $MgCl_2$/0.1 mM EDTA/0.1 mM dithiothreitol/0.4 mM potassium phosphate/150 mM KCl/bovine albumin (0.5 mg/mL)/150 μM CTP, GTP, ATP, and [^{3}H]UTP (0.1 μCi/assay) and the indicated amounts of DNA template as well as RNA polymerase preparation.

Results

The template-primer activity of 5HmUra-containing DNA was compared to that of two conventional DNAs: the host (*Bacillus subtilis*) DNA and calf thymus DNA. The prerequisite for a successful comparison was that the extent of DNase I activation of three types of DNA, i.e., the numbers of strand breaks, was equal. This was the case, as demonstrated in neutral (double-strand breaks) and alkaline (single-strand breaks) gel electrophoresis (results not shown). Maximal template-primer activity of *E. coli* DNA polymerase I was obtained by activation with 5 or 10 ng/mL pancreatic DNase I, and these DNAs were selected for further studies. A relationship between the intensity of DNase treatment and template-primer activity was also confirmed with *Micrococcus luteus* and HL-60 cell DNA polymerases. HL-60 cell DNA polymerase was sensitive to the inhibitory effect of aphidicolin (Table I). This indicated that DNA polymerase α was the prominent polymerase [see, e.g., Zoncheddu et al., (1983)].

The effect of template-primer concentration on the DNA polymerase reactions is illustrated in Figure 1. 5HmUra-containing SPO-1 phage DNA appeared to have the highest template-primer activity for all three polymerases at all template concentrations tested. The lowest activity was noted with *Bacillus subtilis* host DNA. A similar order was obtained when the template-primer activities were tested with different concentrations of *Micrococcus luteus* and *E. coli* DNA polymerases (Figure 2).

Table I: Effect of Aphidicolin on the DNA Polymerase Reaction (HL-60 Cell Enzyme)[a]

template-primer (μg/assay)	[^{3}H]dAMP incorporation (cpm) for co/A[b] in		
	calf thymus	*Bacillus subtilis*	SPO-1
0	158/nt[c]	136/nt	157/nt
3	1388/450	748/361	1374/400
9	1839/403	857/437	1974/398

[a] Six micrograms of HL-60 cell protein. Otherwise, standard reaction conditions. Each figure is the average of two determinations. [b] co, without aphidicolin; A, 0.5 ng/mL aphidicolin. [c] Not tested.

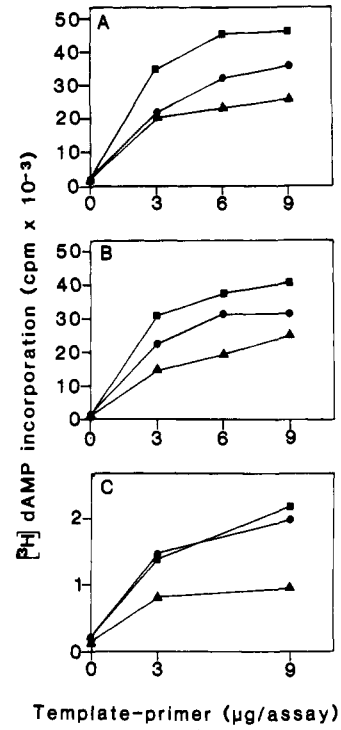

FIGURE 1: Effects of various template-primer concentrations on DNA polymerase reactions. The template-primers were (●) calf thymus DNA, (▲) *Bacillus subtilis* DNA, and (■) SPO-1 phage DNA. (A) *E. coli* DNA polymerase I (0.5 unit), 10 min at 37 °C. (B) *Micrococcus luteus* DNA polymerase (0.1 unit), 10 min at 37 °C. (C) HL-60 cell DNA polymerase (6 μg of protein), 30 min at 37 °C. Each point is the average of two determinations.

The template activity of the three DNAs was also tested with *E. coli* RNA polymerase. For this purpose, nonactivated DNAs were employed. The order of template activity of the three DNAs for this polymerase was similar to the template-primer activity for DNA polymerases; 5HmUra-containing SPO-1 DNA appeared to be the best template (Figure 3).

Discussion

The replacement of Thy by 5HmUra in *Bacillus subtilis* phage SPO-1 shows that 5HmUra can be naturally incorporated into DNA and that its presence per se does not invalidate the various functions of DNA. In fact, it is likely that 5HmUra–DNA provides a selective advantage for SPO-1 phage transcription. This has recently been shown by Choy et al. (1986), who demonstrated that the natural, entirely 5HmUra-containing middle promoter was the most effective as regards RNA polymerase binding and transcription. The

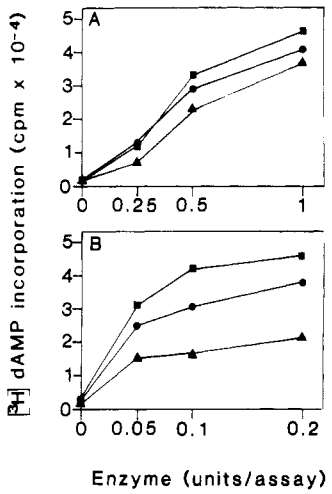

FIGURE 2: Kinetics of replication of three different DNA types with various concentrations of DNA polymerases. (A) *E. coli* DNA polymerase I. (B) *Micrococcus luteus* DNA polymerase. (■) SPO-1 DNA, (▲) *Bacillus subtilis* DNA, (●) calf thymus DNA. The reactions were run with 6 μg of template-primer for 10 min at 37 °C.

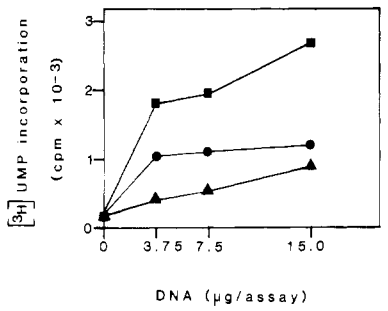

FIGURE 3: Kinetics of transcription of three different DNA types with various concentrations of *E. coli* DNA-dependent RNA polymerase. (■) SPO-1 DNA, (▲) *Bacillus subtilis* DNA, (●) calf thymus DNA. The reactions were run with 0.5 unit of RNA polymerase at 37 °C for 10 min. Each point is the average of two determinations.

present investigation demonstrated that 5HmUra–DNA is a good template-primer for different nonhomologous DNA and RNA polymerases. Hence, it is likely that although more specific mechanisms determine the compatibility of *Bacillus subtilis* and the SPO-1 phage, 5HmUra-DNA may provide a universal advantage for the phage DNA (vs host DNA) in transcription as well as in replication.

The good template-primer activity of 5HmUra-DNA is in sharp contrast to the activity of those template-primers where Thy was replaced by uracil. This kind of situation occurs in *Bacillus subtilis* PBS1-like phages (Takahashi & Marmur, 1963); at least the template-primer activity of uracil-containing deoxyribonucleotide polymers was clearly inferior to that of corresponding Thy polynucleotides when *E. coli* DNA and RNA polymerases were employed as polymerizing enzymes (Vilpo & Ridell, 1986). Furthermore, our results with 5HmUra–DNA differ from those obtained previously with thymine glycol–DNA. Thymine glycol is perhaps the most frequent thymine lesion induced by ionizing irradiation in DNA (Teebor et al., 1988). DNA synthesis was halted progressively throughout thymine glycol containing template DNA when replication in vitro was catalyzed by DNA polymerase I (Hayes & LeClerc, 1986).

The present work demonstrated that 5HmUra–DNA is also a good template-primer for human DNA polymerase. It has been shown previously that administration of 5HmdUrd results in mutations and the induction of SOS functions in bacteria (Bilimoria & Gupta, 1986; Shirname-More et al., 1987), and, in spite of a reported repair mechanism of mammalian 5HmUra–DNA (Hollstein et al., 1984; Boorstein et al., 1987), it has been assumed that the modification of Thy to 5HmUra in normal AT base pairs may be minimally deleterious to mammalian cells (Cannon et al., 1988). This latter conclusion is supported by the present investigation, where good template-primer characteristics of 5HmUra–DNA were demonstrated for different types of DNA and RNA polymerases, including mammalian DNA polymerase α.

5HmUra–protein cross-links have been demonstrated in 5HmdUrd-exposed cells (Matthes et al., 1979). The contribution of this kind of cross-linkage to the toxicity of 5HmdUrd has been questioned, since mammalian cells tolerate high amounts of 5HmUra in their DNA and few, if any, stable 5HmdUrd–amino acid products were formed in [^{3}H]-5HmdUrd-exposed cells (Boorstein et al., 1987). The present data do not allow exclusion of the hypothesis that 5HmdUrd toxicity results primarily from the formation of 5HmUra–protein cross-links (Matthes et al., 1979). Nevertheless, the superior template-primer activity of 5HmUra–DNA, as noted here, suggests that very few, if any, 5HmdUrd–polymerase cross-links were formed.

Although the cytotoxicity of 5HmdUrd has been known for over 30 years, its usefulness in experimental chemotherapy has been appreciated only recently (Kahilainen et al., 1985; Vilpo et al., 1987). The mechanism of action of 5HmdUrd at the cellular level is not known, but we have emphasized the possibility of a negative feedback action by 5HmdUTP, similar to that of dTTP, on the enzyme ribonucleotide reductase (Kahilainen et al., 1986). This may also be the basis of synergism between 5HmdUrd and an important clinical leukemia drug, cytosine arabinoside (Vilpo & Vilpo, 1988). However, we cannot exclude the possibility that 5HmdUrd nevertheless might be deleterious when replacing Thy in DNA.

ACKNOWLEDGMENTS

We thank Leena Vilpo, Tuula Pölönen, and Esko Suvanto for technical assistance.

Registry No. DNA polymerase, 9012-90-2; RNA polymerase, 9014-24-8.

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Generation of a Catalytic Sequence-Specific Hybrid DNase†

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Received March 3, 1989; Revised Manuscript Received June 8, 1989

ABSTRACT: Hybrid nucleases consisting of an oligonucleotide fused to a unique site on the relatively nonspecific phosphodiesterase staphylococcal nuclease have been shown to sequence specifically cleave DNA. We have introduced mutations into the binding pocket of the nuclease which lower the k_{cat}/K_m of the enzyme. Hybrid nucleases generated from these mutants sequence selectively hydrolyze single-stranded DNA in a catalytic fashion, and under a much wider range of conditions than was previously possible. One such hybrid nuclease (Y113A, K116C) was able to site selectively cleave single-stranded M13mp7 DNA (7214 nt), primarily at one phosphodiester bond. Another hybrid nuclease (Y113A, L37A, K116C) catalyzed the hydrolysis of a 78-nt DNA substrate with a k_{cat} of 1.2 min^{-1} and a K_m of 120 nM. The effects of variations in the length and sequence of the oligonucleotide binding region were examined, as were changes in the length of the tether between the oligonucleotide and the enzyme. Cleavage specificity was also assayed as a function of substrate DNA primary and secondary structure and added poly(dA).

One approach to the development of biological catalysts with novel specificities involves the introduction of new binding domains into enzymes. We have previously applied this strategy to the development of sequence-specific nucleases consisting of the relatively nonspecific phosphodiesterases staphylococcal nuclease and RNase S selectively fused to an oligodeoxyribonucleotide of defined sequence via a disulfide linkage. The resulting hybrid nucleases site specifically hydrolyzed both single-stranded DNA and single-stranded RNA adjacent to the oligonucleotide binding site (Corey & Schultz, 1987; Zuckermann et al., 1988; Zuckermann & Schultz, 1988, 1989). These agents, along with those which use other DNA cleaving moieties such as EDTA–Fe(II) (Stroebel et al., 1988; Dervan, 1986), Cu(II)–phenanthroline (Chen & Sigman, 1987), or class IIS restriction enzymes (Kim et al., 1988), are the first steps toward developing restriction enzyme mimics that can cleave RNA or DNA at any desired sequence. Such sequence-specific cleaving agents should have applications in gene cloning, nucleic acid structure studies, and chromosomal mapping.

We now report the effects of alterations in the binding site of staphylococcal nuclease, the length and sequence of the oligonucleotide binding site, and the tether length on the properties of these nucleases. Cleavage specificity as a function of substrate DNA primary and secondary structure and added poly(dA) was also examined. These studies have afforded sequence-specific nucleases *that catalytically cleave single-stranded DNAs* and that function under a wide range of reaction conditions. Moreover, these hybrid nucleases are capable of site specifically hydrolyzing the naturally occurring single-stranded DNA M13mp7. It should be possible to apply a similar combination of chemical and biological mutagenesis to modify the specificity of other classes of enzymes, such as proteases or glycosidases.

Experimental Procedures

General. IR spectra were recorded on a Mattson-Polaris Fourier transform infrared spectrophotometer, and all absorptions were reported in wavenumbers (cm^{-1}). UV spectra were recorded on a Hewlett-Packard diode array 8452A

†Support for this work was provided by the National Institutes of Health (1R01GM41679-01).
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0006-2960/89/0428-8277$01.50/0